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The global nutritional regulator CodY is an essential protein in the human pathogen *Streptococcus pneumoniae*

Stéphanie CAYMARIS^{1,2}, Hester J. BOOTSMA³, Bernard MARTIN^{1,2}, Peter W. M. HERMANS³, Marc PRUDHOMME^{1,2}, Jean-Pierre CLAVERYS^{1,2,@}

¹Centre National de la Recherche Scientifique, LMGM-UMR5100, F-31000 Toulouse, France

²Université de Toulouse, UPS, Laboratoire de Microbiologie et Génétique Moléculaires, F-31000 Toulouse, France

³Laboratory of Pediatric Infectious Diseases, Radboud University Nijmegen Medical Centre, 6500 HB Nijmegen, The Netherlands

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@Correspondence:

Mailing address : Université Paul Sabatier-CNRS, LMGM, Bât. IBCG, 118 route de Narbonne, F-31062 Toulouse Cedex 9, France

e-mail: claverys@ibcg.biotoul.fr; Tel: (33) 561.33.59.11; Fax: (33) 561.33.58.86; <http://www-lmgm.biotoul.fr/uk/equipes/grpclav/>

Summary

CodY is a global regulator highly conserved in low-G+C Gram-positive bacteria. It plays a key role in the adaptation of *Bacillus subtilis* to nutritional limitation through repression of a large gene set during exponential growth and relief of repression upon starvation. In several pathogenic bacteria, CodY regulates major virulence genes. Our interest in *Streptococcus pneumoniae* CodY originates from our observations that the oligopeptide permease Ami was involved in repression of competence for genetic transformation. We hypothesized that peptide uptake through Ami feeds amino acid pools, which are sensed by CodY to repress competence. As our initial attempts at inactivating *codY* failed, we launched an in-depth analysis into the question of the essentiality of *codY*. We report that *codY* cannot be inactivated unless a complementing ectopic copy is present. We obtained genetic evidence that a recently published D39 *codY* knock-out contains additional mutations allowing survival of *codY* mutant cells. Whole genome sequencing revealed mutations in *fatC*, which encodes a ferric iron permease, and *amiC*. This combination of mutations was confirmed to allow tolerance of *codY* inactivation. The *amiC* mutation is in itself sufficient to account for the strong derepression of competence development observed in D39 *codY* cells.

Introduction

CodY is a global regulator highly conserved in low-G+C Gram-positive bacteria (Sonenshein, 2005). In *Bacillus subtilis*, the CodY regulon is large, encompassing nearly 200 genes (Molle *et al.*, 2003), most of which are repressed during exponential growth and induced when cells experience nutrient deprivation. In several pathogenic bacteria, CodY regulates major virulence genes (for review, see Sonenshein, 2005; Sonenshein, 2007). The repressor function of CodY (i.e. its DNA-binding activity) is activated by interaction with branched-chain amino acids (BCAAs), as originally shown *in vivo* in *Lactococcus lactis* (Guédon *et al.*, 2001). In species other than the Streptococci, Lactococci and Enterococci, CodY is also activated by interaction with GTP as first demonstrated (Ratnayake-Lecamwasam *et al.*, 2001) and further documented (Handke *et al.*, 2008) with the *B. subtilis* protein. In the latter bacterium, CodY is known to control not only metabolic pathways but also cellular processes, such as motility, sporulation, and competence for genetic transformation (Sonenshein, 2005; Sonenshein, 2007). In the latter case, CodY represses both *comK*, which encodes the master transcriptional activator of competence (*com*) genes, and *srfA*, a key operon for transcriptional activation of *comK* (Serror and Sonenshein, 1996).

Until recently, little was known regarding the role(s) of CodY in *Streptococcus pneumoniae*. Our interest in CodY stems from our previous observations that the oligopeptide permease Ami was, together with its dedicated oligopeptide-binding lipoproteins (Obl, i.e. AmiA, AliA, and AliB) (Alloing *et al.*, 1994), indirectly involved in the regulation of competence (Claverys *et al.*, 2000; Claverys and Håvarstein, 2002). Competence development of an *obl* mutant thus occurred at a ~50-fold reduced cell density compared to wild type (Alloing *et al.*, 1998). We hypothesized that the uptake of oligopeptides plays a key role in metabolic regulation in *S. pneumoniae*, by providing information on nutrient availability

(Claverys *et al.*, 2000). Exhaustion of nutrients would be sensed through a mechanism involving peptide uptake by the Ami-Obl oligopeptide permease, followed by peptidase digestion to release amino acids (aa) leading to replenishment of aa pools, which in turn affect a global regulatory protein such as CodY (Claverys *et al.*, 2000). The above-mentioned demonstration that BCAAs are effectors of CodY in several species would fit nicely with this hypothesis since *S. pneumoniae* Obl proteins have different but overlapping specificities for peptides including those containing BCAAs (Alloing *et al.*, 1994). Thus import of BCAAs (as part of oligopeptides) by the Ami-Obl permease could directly impact CodY activity. This situation would not be unprecedented since CodY-dependent repression of *comK* and *srfA* was lost in an *opp* (oligopeptide permease) mutant of *B. subtilis* (Serror and Sonenshein, 1996). To account for the negative impact of Ami-Obl on competence induction, we explicitly stated in our model that CodY should repress competence (Claverys *et al.*, 2000).

To test this model, we initiated experiments aimed at inactivating *S. pneumoniae codY*. Surprisingly, this gene turned out to be difficult to inactivate. However, *codY* inactivation had recently been achieved through insertion of a trimethoprim resistance gene (*trim*) in strain D39 (Hendriksen *et al.*, 2008). Microarray analysis using this mutant suggested that pneumococcal CodY functions mainly as a transcriptional repressor, as 43 of the 47 genes differentially expressed in the *codY::trim* mutant were found to be upregulated. The inefficiency in constructing a *codY* knock-out in strain R6 prompted us to launch an in-depth analysis of the question of the essentiality of *codY*. Here, we present our data establishing that *codY* is essential in our laboratory strains, as well as in *S. pneumoniae* R6 and encapsulated D39 strains. We provide genetic evidence that, in addition to the *codY* knock-out, the *codY::trim* mutant used for transcriptome analysis of the CodY regulon and for virulence studies (Hendriksen *et al.*, 2008) contains additional mutations (suppressors) allowing survival of *codY* mutant cells. We document a strong derepression of competence in such

codY-mutant cells. Finally, we use comparative whole genome sequencing to identify suppressor mutations in *fatC*, which encodes a ferric iron permease, and *amiC*. We confirm that this combination of mutations allows tolerance of *codY* inactivation and discuss possible reasons for CodY essentiality in *S. pneumoniae* in light of these observations.

Results

Minitransposon insertion mutagenesis of S. pneumoniae codY is not possible

A *codY* PCR fragment amplified with the primer pair MP188-MP189 (Table 1) was used as target for *mariner* mutagenesis (Experimental procedures). Analysis of more than 50 minitransposon insertions through PCR revealed that only 6 clones produced an MP188-MP189 fragment of which the size was consistent with that predicted for integration of the *kan^R* minitransposon (donated by plasmid pR410; Table 1). Localization of these 6 insertions indicated that none of them had inserted into the *codY* gene (Fig. 1A). Given our previous experience with *mariner* mutagenesis of the *ciaRH* (Martin *et al.*, 2000), *endA*, *comEAC*, *comFAC*, *comGAB*, and *dprA* (Bergé *et al.*, 2002), as well as *cibABC* (Guiral *et al.*, 2005), *comM-lytR* (Håvarstein *et al.*, 2006) and *radC* (Attaiech *et al.*, 2008) loci, we concluded that the failure to isolate insertions in a locus occupying a central position on the targeted PCR fragment and the biased distribution observed were strongly indicative of the essentiality of *codY*, at least in the genetic background and under the plating conditions (CAT-agar) used. [It is of note that a similar conclusion regarding the essentiality of *lytR* (*spr1759*) based on the failure to isolate *mariner* insertions in this locus (Håvarstein *et al.*, 2006) was confirmed in a further study (Johnsborg and Havarstein, 2009).]

Construction of a strain harboring a second (ectopic) copy of *codY*

The failure to inactivate *codY* through *mariner* mutagenesis prompted us to construct a strain harboring a second copy of *codY* that should tolerate inactivation of one of the two copies. The second copy was inserted at CEP (chromosomal expression platform) (Guiral *et al.*, 2006) under the control of the maltose-inducible promoter, P_M (Fig. 1B). The resulting strain is referred to as *codY*^{+/+} hereafter. The duplication of *codY* had no detectable effect on growth and spontaneous competence induction when cells were grown with 1% maltose, i.e. under conditions leading to full induction of the P_M promoter (Guiral *et al.*, 2006), suggesting that the level of CodY attained with this induction system is not detrimental to the cell (data not shown).

Minitransposon insertions in *codY* are readily obtained in diploid (*codY*^{+/+}) cells

A *codY*^{+/+} strain (R2349) was then used as recipient for *mariner* mutagenesis of *codY* with plasmid pR412 as donor of minitransposon (*spc*^R cassette; Table 1). In contrast to the failure to inactivate *codY* using wildtype recipient cells, minitransposon insertions were readily obtained in cells grown in maltose. A *codY*1-*codY*2 (Table 1) PCR fragment of which the size was consistent with that predicted for integration of the *spc*^R minitransposon was observed for 11 out of 20 randomly selected clones. Five of these insertions turned out to inactivate *codY* as judged from their location (Fig. 1C) and from the inability to introduce them at high frequency in a wildtype recipient (data not shown but see next section).

The isolation of minitransposon insertions in the genuine copy of *codY* suggested that the ectopic copy placed at CEP was able to complement *codY* deficiency. This conclusion was further strengthened by the observation that upon transformation of *codY*^{+/+} cells with a PCR fragment carrying the *codY*::*spc*^{3A} insertion (generated with the primer pair *codY*atg-*codY*stop; Fig. 1C and Table 1), *Spc*^R transformants distributed about equally between the genuine *codY* (e.g. strain R2424 the genotype of which is denoted *codY*^{*spc3*+/+}; Fig. 2A) and the ectopic CEP_M-*codY* loci (e.g. strain R2425 the genotype of which is denoted *codY*^{+/+*spc3*}; Fig. 2B).

Transformation frequencies of codY^{spc3}+/+ in wildtype and codY+/+ cells confirm codY essentiality

To unambiguously demonstrate the essentiality of *codY* in our strains, we then used chromosomal DNA from a *codY*^{*spc3*+/+} strain (R2438) as donor in transformation of a wildtype recipient. If *codY* is essential, the survival of transformants harboring the *codY*::*spc*^{3A} knock-out mutation is predicted to rely on the simultaneous integration of CEP_M-*codY*-*kan* (i.e. a double transformation event occurring independently as the two loci are not genetically linked). On the other hand, a *codY*^{+/+} recipient should readily accept the *codY* knock-out. Transformation frequency should therefore be significantly reduced in wildtype cells compared to a *codY*^{+/+} recipient. In full agreement with this prediction, transformation of *codY*::*spc*^{3A} occurred in *codY*^{+/+} (strain R2350) cells with a frequency close to that of the *str41* (*Str*^R) reference marker [Note that transformation of the *spc*^R cassette, which requires integration of ~1.1 kb heterologous DNA, is expected to occur with a 2 to 3-fold reduced frequency compared to the point-mutation reference marker *str41*] (Fig. 3A). In contrast, ~20-

fold reduction in the frequency of Spc^R transformants was observed with wildtype (R895) cells (Fig. 3A). The observed frequency was close to that calculated as the product of individual transformation frequencies of Spc^R and Kan^R . To verify that Spc^R transformants obtained with the wildtype recipient had simultaneously acquired resistance to Kan, 10 Spc^R transformants were isolated. All of them turned out to carry the $\text{CEP}_M\text{-codY-kan}$ construct (data not shown). Altogether, these data demonstrated that *codY* is an essential gene in *S. pneumoniae* R800 and its derivatives.

codY is also essential in R6 and in encapsulated D39

R800 was originally derived from R6 through introduction of a suppressor mutation (originating from strain Cl₃, another D39 derivative) (Tiraby *et al.*, 1975) that greatly improved growth of *ami* mutants (Lefèvre *et al.*, 1979). This led us to wonder whether the presence of this uncharacterized suppressor was responsible for the essentiality of *codY*. We therefore introduced the $\text{CEP}_M\text{-codY}$ construct in R6 (generating strain R2427) and measured transformation frequencies of $\text{codY}::\text{spc}^{3A}$ using $\text{codY}^{\text{spc3/+}}$ chromosomal DNA as donor. Yield of Spc^R transformants was significantly lower in R6 than in its $\text{codY}^{+/+}$ derivative indicating that *codY* is essential in this background as well (Fig. 3B).

Inactivation of *codY* was previously reported in the encapsulated *S. pneumoniae* D39 strain (Hendriksen *et al.*, 2008). To check whether our negative data could be explained by the use of unencapsulated strains, of different medium and/or plating conditions, we first tried to transform D39 with an *amiF1-kan1* PCR fragment carrying $\text{codY}::\text{spc}^{3A}$ (Fig. 2B), using previously described conditions for plating of *codY*⁻ transformants on Columbia base agar (Hendriksen *et al.*, 2008). No Spc^R transformants (i.e. $<10^{-6}$) could be obtained (data not

shown). We therefore adopted the same strategy as the one described above for R6. Transformation of *codY::spc^{3A}* using *codY^{spc3/+}* chromosomal DNA as donor yielded ~20-fold fewer *Spc^R* transformants in D39 than in its *codY^{+/+}* derivative (strain TD73) indicating that introduction of a *codY* knock-out in D39 also requires the simultaneous transfer of the complementing ectopic CEP_M-*codY* gene (Fig. 3C). In full agreement with this interpretation, *Spc^R* transformants were also Kan^R. We concluded that *codY* is also essential in encapsulated D39 and that this gene must therefore be added to the list of essential pneumococcal genes (Thanassi *et al.*, 2002).

Re-investigation of the previously described codY knock-out reveals the presence of additional suppressor mutations or a chromosomal rearrangement

We then re-examined the previously constructed *codY::trim* mutant, strain D39 Δ *codY* (Hendriksen *et al.*, 2008), as the transformation frequency upon mutant generation was very low (W. Hendriksen, personal communication). PCR analysis of the *codY* chromosomal region of strain D39 Δ *cps* Δ *codY*, which was derived from D39 Δ *codY* by transformation with a PCR fragment harboring the Δ *cps::kan* cassette, as previously described (Bootsma *et al.*, 2007), confirmed the published structure (data not shown). Preliminary attempts using D39 Δ *cps* Δ *codY* chromosomal DNA as donor revealed that transformation of the *codY::trim* construct into R800 derivatives occurred only at very low frequency (data not shown). To facilitate the comparison with transformation experiments reported above, we replaced the *codY::trim* construct with the *codY::spc^{3A}* insertion (using as donor a *codY*1-*codY*2 PCR fragment) and we introduced the *str41* reference marker (using as donor a PCR fragment amplified with the rpsL_3-rpsL_4 primer pair; Table 1) thus generating strain TD81. Then,

using TD81 chromosomal DNA as donor, we compared transformation frequencies of *codY::spc^{3A}* in the same pairs of *codY⁺* and *codY^{+/+}* derivatives of R800, R6 and D39 used in Fig. 3. Transformation frequencies in *codY⁺* strains were reduced by 544, 762, and 351 fold, respectively in R800, R6, and D39, compared to their *codY^{+/+}* isogenic derivatives (Fig. 4A-C). These very large reductions in transformation frequency (compared to the ~20-fold reduction observed when the simultaneous transfer of *codY::spc^{3A}* and CEP_M-*codY* from a *codY^{spc3/+}* donor was required; Fig. 3) indicated that transfer of the *codY::spc^{3A}* from strain TD81 must be accompanied by the transfer of more than one point mutation. Alternatively, the very low transformation frequency could be accounted for by the requirement for a simultaneous chromosomal rearrangement. We concluded that the original D39Δ*cps*Δ*codY* strain either contained two additional suppressor mutations that are presumably acting together to compensate for the absence of CodY and restore cell viability, or harbored a chromosomal rearrangement allowing survival in the absence of CodY. We tentatively named it/them *socY* (for suppressor of codY).

To get a possible insight into the molecular nature of the mutation(s) involved, we carried the same transformation experiments but with a pair of *hex* mutant derivatives of R800 (strain R1818 and its *codY^{+/+}* derivative, R2428). The Hex system of *S. pneumoniae* is known to correct out some mismatches at the donor-recipient heteroduplex stage in transformation (Claverys and Lacks, 1986). It is particularly efficient at correcting transition mismatches (i.e. A/C or G/T) as well as short frameshifts (Gasc *et al.*, 1989). Transformation frequencies with *hex⁻* recipient cells were improved ~4.5 fold (125 versus 544 fold reduction in the *codY⁺* parent compared to the *codY^{+/+}* derivative, in *hex⁻* and *hex⁺* strains respectively; Fig. 4D), which suggests that one of the *socY* mutations is recognized by the Hex system and is therefore possibly a transition or a short frameshift. Alternatively, the recombination event

leading to the putative chromosomal rearrangement may involve the formation of a heteroduplex intermediate harboring mismatches susceptible to the Hex system.

Effect of codY inactivation on spontaneous competence development

To characterize the role of *S. pneumoniae* CodY with respect to growth and the regulation of competence, we first tried to deplete CodY making use of a *codY*^{spc/+} strain, i.e. a strain in which the only functional copy of *codY* was under the control of the P_M promoter. In light of the failure to obtain a functionally significant depletion of CodY (Supplementary results), we chose to characterize a *codY socY* strain with respect to spontaneous competence development and growth. Competence was monitored throughout incubation at 37°C by using a transcriptional fusion of the *luc* gene, which encodes luciferase, to the *ssbB* gene. The latter is known to be specifically induced at competence. The *ssbB::luc* fusion thus reports on competence through light emission by luciferase (Prudhomme and Claverys, 2007). We introduced the *ssbB::luc* transcriptional fusion (using R895 chromosomal DNA as donor) in strains D39Δ*cps* and D39Δ*cps*Δ*codY socY* thus generating strains TD82 and TD83, respectively (Table 1). We then compared competence profiles of strains TD82 and TD83 during growth in C+Y medium with initial pH values between 6.48 and 7.26, since spontaneous competence induction is known to be strongly dependent on the initial pH. For instance, initial pH values between 6.8 and 8.0 affected the timing of occurrence and the level of competence (Chen and Morrison, 1987). While the wildtype parent developed spontaneous competence only in cultures with initial pH values above 7.0 (Fig. 5A-B), the *codY* mutant could develop competence under acidic conditions, down to an initial pH value of 6.70 (Fig. 5C-D). It is of note that despite the presence of the *socY* suppressor, *codY* mutant cells grew

more slowly than wildtype cells in C+Y medium (Fig. 5A-D). *codY* mutant cells thus entered the stationary phase of growth after ~270 min incubation compared to ~170 min for wildtype cells. This >50% increase in generation time might indicate that CodY plays an important role in the regulation of pneumococcal growth. Despite the fact that *codY* mutant cells grew more slowly than wild type, they developed competence at about the same time (e.g. maximum competence after 87 versus 82 min incubation at pH 7.26; Fig. 5A and 5C), which corresponded to OD₄₉₂ values of 0.083 and 0.115, respectively. This observation was also consistent with upregulation of competence in the *codY* mutant. The strong competence-upregulated (*cup*) phenotype (Martin *et al.*, 2000) displayed by *codY* mutant cells would be consistent with the hypothesis that CodY normally represses competence under acidic conditions in wildtype cells. However, the uncharacterized suppressor mutations (*socY*) in strain TD83 could also be responsible for the observed *cup* phenotype. This prompted us to investigate the phenotype of *socY* (*codY*⁺) cells.

Impact of socY on spontaneous competence development

First, to establish whether restoration of *codY*⁺ would be tolerated in a *socY* genetic background, strain TD83 was transformed with R2644 chromosomal DNA. R2644 carries the *spc*^{90C} insertion immediately upstream of the *codY*⁺ gene (see Experimental procedures). Integration of the *spc*^R cassette by transformation could thus be accompanied by the removal of the adjacent *codY::trim* cassette. A failure to survive of Trim^S (i.e. *codY*⁺) excisants should result in a drastic reduction in the number of Spc^R transformants, since recombination events leading to *spc* integration without the simultaneous deletion of *trim* are rare due to the reduced distance between the two cassettes (105 bp). The high Spc^R to Str^R transformant ratio

observed (average value 0.32 ± 0.06) was consistent with the simultaneous occurrence of both integration (of the *spc* cassette) and excision (of the *trim* cassette) events, and suggested that *socY* (*codY*⁺) cells are viable. To establish this, 4 of 4 randomly chosen *Spc*^R transformants were first checked to be *Trim*^S. Then, they were shown to readily re-accept *codY::trim* when transformed with TD80 chromosomal DNA (*Trim*^R/*Str*^R average ratio of 0.25 ± 0.08 over 15 independent cultures). These data confirmed that the *Spc*^R *Trim*^S transformants were still *socY* and indicated that none of the two suppressor mutations or the putative chromosomal rearrangement is detrimental to pneumococcal cells when *CodY* is present. One of the *Spc*^R *Trim*^S clones was retained and named strain TD95. The *spc*^{90C} cassette was then removed from strain TD95 by transformation with a PCR fragment generated on R800 chromosomal DNA with the MP188-MP189 primer pair, followed by phenotypic expression and segregation in liquid culture (C+Y medium) for 4.5 hours, and plating on CAT-agar without antibiotic. Individual colony screening was carried out to isolate a *Spc*^S clone, TD96. Upon transformation of TD96 with TD80 chromosomal DNA, a *Trim*^R/*Str*^R ratio of 0.42 ± 0.07 was observed indicating that TD96 had remained *socY*.

The competence profile of TD96 during growth in C+Y medium with initial pH values between 6.48 and 7.26 was compared with that of TD82 (wild type) and TD83 (*codY::trim socY*). TD96 *socY* (*codY*⁺) cells grew more rapidly than parental *codY::trim socY* cells, but still more slowly than wild type (entry into stationary phase after ~210 min versus ~170 min; Fig. 5E-F). Interestingly, *socY* cells displayed a stronger *cup* phenotype than *codY socY* cells as judged first from their ability to develop spontaneous competence at pH 6.60 (Fig. 5F) and second, from the very early development of competence (36 min at pH 7.26) compared to both wildtype and *codY socY* cells (Fig. 5). Thus, the *socY* mutations alone confer a strong *cup* phenotype. The net effect of *codY* inactivation in this background is to attenuate this *cup*

phenotype. From these data, it is therefore difficult to conclude that CodY acts as a repressor of competence in pneumococcal cells.

Whole genome sequence comparison suggests fatC and amiC mutations suppress inviability of codY mutants

In an attempt to identify the *socY* (suppressor) mutations, whole genome resequencing of strains D39 and D39 Δ *codY* was performed (Experimental procedures). Compared to the previously published D39 NCTC 7466 genome (Lanie *et al.*, 2007), the D39 Δ *codY* strain had 14 mutations also found in its parent. As our D39 was obtained originally from NCTC (via T. Mitchell), these mutations presumably have arisen during laboratory cultivation. None of them appeared to alter an important function (Table S1), which is consistent with the fact that this D39 strain displayed full virulence in a mouse model (Hendriksen *et al.*, 2008).

The *codY* mutant had also mutations flanking the *trim* cassette [a C \rightarrow T transition, a GT \rightarrow AGC frameshift and a T \rightarrow A transversion, respectively at positions -175, +681/682 and +724 (positions are given with respect to the ATG of *codY*)]. All these changes occurred in between the *trim* cassette and the oligonucleotide primers used to amplify the *codY* region (Hendriksen *et al.*, 2008), and were presumably introduced during polymerase chain reaction. The transition mutation is in itself sufficient to lower the frequency of integration of the *codY::trim* cassette by ~5-fold during transformation of mismatch-repair proficient strains.

Most relevant with respect to the question of CodY essentiality, the *codY* mutant had, in addition, a mutation in *fatC* and a second, variable mutation in *amiC* (Fig. 6A). The former mutation (a C \rightarrow T transition), *fatC*^{C496T} changed a CAA (Gln) codon into TAA (stop) in a gene belonging to the *fatD-fatC-fecE-fatB* operon (*spd_1649-1652* in D39; *spr1684-1687* in R6;

339 *sp1869-1872* in TIGR4) (Fig. 6A, top). This operon [also called *piuBCDA* or *pit1* (Brown *et*
340 *al.*, 2002)] encodes the major ferric iron/heme [*fatB* has been shown to bind heme (Tai *et al.*,
341 2003)] transporter of *S. pneumoniae* (Ulijasz *et al.*, 2004). The *fatC*^{C496T} change is predicted to
342 result in the synthesis of a truncated FatC protein (165 instead of 318 aa) and was detected in
343 100% of forward and reverse reads. As concerns the base variations in *amiC*, which encodes a
344 598-aa protein and belongs to the *amiACDEF* operon (*spd_1671-1667* in D39; *spr1707-1703*
345 in R6; *sp1891-1887* in TIGR4), three types of mutations were observed: two single-base
346 changes (#2 and #3 in Fig. 6A, bottom) and a more complex mutation (#1 in Fig. 6A, bottom).
347 These variations were detected in only a fraction of sequence runs. Mutations #2 (*amiC*^{G1438T})
348 and #3 (*amiC*^{G1459T}) change a GAC (Asp) codon into TAC (Tyr) (aa 480 and 487,
349 respectively); these mutations were detected in 30.8% of forward and 11.1% of reverse reads
350 (total with variation: 19%), and in 57.1% of forward and 40.9% of reverse reads (total with
351 variation: 47%), respectively. Interestingly, both changes affect an Asp aa conserved in the
352 entire family of Opp (oligopeptide), Dpp (dipeptide) and App (nickel) permeases (our
353 observations), which suggests that they are functionally important and that the corresponding
354 AmiC proteins may have lost their activity. The complex mutation #1 consisted of a G→TT
355 frameshift (*amiC*^{G246TT}) and the almost adjacent A→C change (*amiC*^{A248C}); both mutations
356 shared the same sequencing parameters (detection in 25.0% of forward and 33.3% of reverse
357 reads), which strongly suggests that they resulted from a single mutational event (total with
358 variation, 29%). This mutation could thus be described as a GGA→TTGC change. [In fact,
359 this change had already been observed during analysis of mismatch repair specificity in *S.*
360 *pneumoniae* and referred to as *amiA29* (at this time, the *ami* locus was thought to be a single
361 gene, hence the name *amiA*) (Gasc *et al.*, 1989) (for a molecular explanation of this
362 mutational event, see Fig. S2).] This complex mutation is predicted to result in the synthesis

of a truncated protein harboring the first 81 aa of AmiC fused to 13 ‘new’ aa (resulting from the frameshift).

At first sight, the presence of changes #1, #2 and #3 in only a fraction of sequence runs appeared puzzling and suggested their late occurrence in an original $\Delta codY$ $fatC^{C496T}$ clone. As a first attempt to establish whether an *ami* mutation was required for tolerance of *codY*, we analyzed the *ami* locus of D39 $\Delta cps\Delta codY$, considering it a subclone of the original D39 $\Delta codY$ strain since it was generated by transformation of this strain with a PCR fragment harboring the $\Delta cps::kan$ cassette. Taking advantage of the fact that *amiC-F* mutations normally confer resistance to methotrexate (Mtx^R), transformation of an Mtx^S strain (R1501) with a series of PCR fragments covering the *ami* region of strain D39 $\Delta cps\Delta codY$ was used to localize any *ami* mutation (Fig. S3). Transformation data were consistent with the presence of mutation #2 or #3 in strain D39 $\Delta cps\Delta codY$; DNA sequencing confirmed that this strain had inherited the latter mutation (*amiC*^{G1459T}). These data prompted us to directly confirm the mixed structure population with respect to *amiC* of the original D39 $\Delta codY$ strain. An aliquot of the original stock was plated, 16 individual clones were picked and *amiC* (and *fatC*) regions were sequenced (Table S2). All clones turned out to harbor one of the three *amiC* mutations identified during whole genome sequencing (and 11 out of 11 clones sequenced carried the *fatC* mutation). Interestingly, the overall distribution between the three types was in very good agreement with that inferred from the analysis of genome sequence data (Table S2). It is also of note that 3 clones harbored change #1 (i.e. both *amiC*^{G246TT} and *amiC*^{A248}), which provided support to the view that a single molecular event accounts for a complex change (Fig. S2). Altogether, these results strongly suggested that inactivation of *amiC* was required for tolerance of *codY* inactivation. We tentatively concluded from these observations that the combination of *fatC* and *amiC* mutations suppresses inviability of *codY* mutants.

388

389 *The fatC amiC combination allows tolerance of codY inactivation*

390

391 To confirm this conclusion, we generated *mariner* insertion mutants in *fatC* (Fig. S4). Then,
392 taking advantage of the availability of the well-characterized *amiC9* mutation (*amiC*^{C355T})
393 [previously named *amiA9* (Gasc *et al.*, 1989)] which changes a CAA codon (Gln) into a TAA
394 (stop) codon leading to the synthesis of a truncated AmiC protein (118 aa), we investigated
395 the ability of recipient cells harboring the *fatC::cat*^{23C} cassette and/or the *amiC9* mutation
396 (Fig. 6A) to accept *codY* inactivation.

397 Strain (R246) and its *amiC9* (R3003), *fatC::cat*^{23C} (R3002) and *amiC9 fatC::cat*^{23C}
398 (R3004) derivatives were used as recipients for the *codY::trim* or *codY::spc* cassettes,
399 respectively carried on TD80 and TD81 chromosomal DNA. These recipients contained a
400 *hexA*⁻ mutation, which impairs mismatch repair, firstly to ensure similar integration frequency
401 of the cassettes since transfer of the *trim* cassette is otherwise predicted to be reduced by the
402 Hex system due to the presence of flanking mismatches (see above); secondly to equalize
403 transformation frequencies of *amiC*^{G1459T} and *fatC*^{C496T} point mutations.

404 The *amiC fatC* double mutant derivative readily accepted the *codY* (*spc* or *trim*) cassette
405 with the expected frequency relative to the reference marker *str41* (~0.20), while introduction
406 of the cassette into its wildtype parent occurred with a ~45-fold reduced frequency (Fig. 6B).
407 As expected, *amiC* and *fatC* single mutants displayed a ~5 to 10-fold reduction in
408 transformation frequency of the *codY* cassette compared to *amiC fatC* cells. The latter
409 reduction reflected the need for co-transformation of the *fatC::cat*^{23C} and *amiC9* mutation,
410 respectively, while co-transformation of both *amiC* and *fatC* together with the *codY* cassette
411 was required in wildtype cells. Altogether, these results demonstrated that the *amiC fatC*
412 combination fully suppressed the inviability of *codY* mutant cells.

Growth and spontaneous competence development of wild type (TD82) and *amiC9* (TD84), *fatC::cat^{23C}* (TD138), *amiC9 fatC::cat^{23C}* (TD135) and *amiC9 fatC::cat^{23C} codY::trim* (TD154) mutant strains were then compared (Fig. S5). Both *fatC* and *amiC* mutant strains displayed a *cup* phenotype, consistent in the latter case with the phenotype of *obl* mutants (Alloing *et al.*, 1998). The *cup* phenotype of the double mutant was most similar to that observed with the *amiC* mutant (Fig. S5) and with the *socY* strain (Fig. 5E-F). Finally, while *codY* inactivation resulted in slower growth confirming the observation in Fig. 5, it did not significantly attenuate the *cup* phenotype observed with the *amiC fatC* double mutant (compare TD135 and TD154, Fig. S5).

Discussion

CodY is a pleiotropic regulator in low-GC Gram-positive bacteria involved in the control of different processes like aa uptake, competence, sporulation and virulence. We first established that *codY* is an essential gene in the D39 strain of *S. pneumoniae* and in several of its laboratory derivatives by using different genetic approaches (*mariner* mutagenesis, complementation, transfer of mutations in different genetic backgrounds; Fig. 1-3). We then showed that a previously obtained *codY* mutant (D39 Δ *codY*) used to identify the CodY regulon (Hendriksen *et al.*, 2008) contains additional suppressor mutations, called *socY*, one of which was concluded to correspond to a transition mismatch or a frameshift (Fig. 4). We also showed that both the *codY socY* and *socY* strains displayed a competence upregulated or *cup* phenotype (Fig. 5).

Whole genome sequencing of strains D39 and D39 Δ *codY* undertaken to identify the *socY* suppressors revealed the presence of mutations in *fatC* and *amiC*, which encode the

membrane permease component of ferric iron/heme and oligopeptide ABC transporters, respectively (Fig. 6A). This combination of mutations was unambiguously demonstrated to be required to allow tolerance of *codY* inactivation (Fig. 6B). These results fully confirm the conclusions from *codY* transfer experiments, including the presence of a mutation susceptible to mismatch repair (Fig. 4D), i.e. the *fatC*^{C496T} transition.

Proposed scenario for the initial stabilization/survival of D39ΔcodY cells

Genome sequence clearly indicated that the *fatC* mutation arose first. However, genome sequence data also strongly suggested that inactivation of *amiC* is absolutely required for tolerance of *codY* inactivation. The finding that three different *amiC* mutations occurred in the D39Δ*codY* strain, as confirmed by individual subclone analysis (Table S2), implied a strong selection pressure (growth advantage) for *amiC* mutant derivatives. The *amiC* mutations presumably arose independently in an otherwise *codY fatC* lineage.

It is of note that the D39Δ*codY* strain described in Hendriksen *et al.* (2008) was obtained by backcrossing D39 using chromosomal DNA isolated from an initial D39Δ*codY::trim* transformant. Despite this careful strategy, D39Δ*codY::trim* turned out to contain two additional mutations, as shown in this study. While a second round of transformation normally eliminates unlinked mutations, it is obviously unable to do so when the unlinked mutations are absolutely required for survival. In addition, we noticed that the two suppressor mutations are not very distant. The two closest mutations (*fatC*^{C496T} and *amiC*^{G1459T}), located 17,987 nt apart, could frequently be carried by the same DNA fragment. Careful comparison of transformation frequencies with a well-characterized reference marker (preferably present

on the same chromosomal DNA) is thus the only way to establish unambiguously that any construct is well tolerated and does not affect cell viability.

CodY, socY and competence

It was implicit in our working hypothesis connecting competence regulation to oligopeptide uptake via a global regulator (like CodY) sensing aa pools (see Introduction) that this regulator would act as a repressor of competence (Claverys *et al.*, 2000). Our data rather suggest that, in contrast to this expectation, inactivation of *codY* has no major effect on spontaneous competence, most of the effects seen being attributable to the *socY* mutations (Fig. 5 and Fig. S5). In fact, the *amiC* mutation is in itself sufficient to account for the strong derepression of competence development observed in D39 Δ *codY* cells (Fig. S5). It is of note that despite the *cup* phenotype documented in this study for the D39 Δ *cps* Δ *codY* strain (Fig. 5), previous transcriptome analysis did not reveal induction of any *com* gene (Hendriksen *et al.*, 2008); presumably, culture conditions used for mRNA extraction did not lead to differential induction of the *com* regulon between the *codY* mutant and its parent, competence being either similarly induced or repressed for both strains. While normal Ami functioning somehow leads to competence repression, CodY is either neutral (Fig. S5) or possibly acts as an activator of competence, the latter conclusion being suggested by the attenuation of the *cup* phenotype of *amiC fatC* cells upon introduction of the *codY* knock-out (Fig. 5). If confirmed, the role of *S. pneumoniae* CodY with respect to competence regulation would thus differ from that of its *B. subtilis* orthologue, which represses competence (Serror and Sonenshein, 1996), suggesting that the impact of nutrient deprivation on competence induction is opposite in *B. subtilis* and *S. pneumoniae* or at least that nutritional signals are conveyed in a very different way in these

two species. However, since the impact of *codY* inactivation on competence could so far be evaluated only in complex genetic backgrounds (i.e. mutant for both *amiC* and *fatC*), further work using different approaches, such as a transient depletion of CodY, would be necessary to evaluate the exact role of CodY in the regulation of pneumococcal competence.

CodY, socY and previous transcriptome studies

The finding that the D39 Δ *codY* strain used in a previous study (Hendriksen *et al.*, 2008) was in fact a *codY amiC fatC* triple mutant raises the question of the respective contribution of each mutation to the phenotypes previously attributed solely to the inactivation of *codY*. As concerns transcriptome data, upregulation of *aliA* (*aliB* was also upregulated but only 1.8-fold), *amiA-amiC-amiD* and *fatD-fatC-fecE-fatB* was reported in Δ *codY* (Hendriksen *et al.*, 2008). Binding of CodY to P_{*amiA*} (as well as to P_{*aliB*}) was consistent with a direct regulation of oligopeptide uptake by CodY. CodY thus exhibited a strong affinity for P_{*amiA*} although the effect of BCAA addition was limited (1.56-fold versus 2 to 20-fold enhancement for other promoters). On the other hand, the *K_d*s (the CodY concentration at which 50% of the fragment is shifted) for the P_{*fatD*} promoter was higher than the highest concentration tested (2,000 nM) indicative of a rather weak affinity and was unaffected by addition of BCAAs, contrary to most other CodY-regulated promoters (Hendriksen *et al.*, 2008). Possibly, the binding of CodY to P_{*fatD*} is affected in the presence of other regulators (see below), as *fat* regulation is clearly multifactorial (Ulijasz *et al.*, 2009). Alternatively, since Δ *codY* cells used to prepare mRNA for transcriptome analysis were also mutant for *fatC* (i.e. they lacked the main iron transporter of *S. pneumoniae*), the upregulation of the *fatD-fatC-fecE-fatB* operon could be a reflection of (inefficient) attempts at iron homeostasis by increasing expression of

this transporter rather than the consequence of a lack of repression in the absence of CodY. The same reasoning may apply to *dpr* gene expression. This gene (*spd_1402* in D39; *spr1430* in R6; *sp1572* in TIGR4) encodes a conserved iron storage-peroxide resistance protein (Ulijasz *et al.*, 2004) that is possibly essential (Pericone *et al.*, 2003). It was found to be downregulated in the *codY* mutant (Hendriksen *et al.*, 2008). Depletion of iron in *codY* mutant cells resulting from *fatC* inactivation could possibly account for the observed reduction in *dpr* expression without implying any direct regulation by CodY. This would be consistent with the failure to identify a sequence resembling the CodY box upstream of the *dpr* gene. Clearly, additional work is necessary to establish whether CodY regulates *dpr* expression directly or indirectly.

CodY, socY and previous virulence studies

Similarly, the presence of *amiC* and *fatC* mutations in the D39 Δ *codY* strain used in a previous study (Hendriksen *et al.*, 2008) raises the question of their respective contribution to the virulence phenotypes previously attributed solely to *codY* inactivation. The importance of iron for bacterial growth and virulence is well established. Thus, signature-tagged mutagenesis (STM) screens for genes essential during pneumococcal pneumonia identified insertions in genes belonging to each of the three iron transporters characterized in *S. pneumoniae* (Brown *et al.*, 2001; Brown *et al.*, 2002), *piuB* (i.e. *fatD*), *pitB* (*pitADBC* operon) and *piaA* (*piaBCD* operon) (Hava and Camilli, 2002). These iron uptake systems have been demonstrated to be important for full pneumococcal virulence, especially upon simultaneous mutation of two operons (Brown *et al.*, 2001; Brown *et al.*, 2002). Single mutation of *piuB* resulted in only a

mild reduction of virulence in a pneumonia model, while no attenuated phenotype was observed in systemic infection (Brown *et al.*, 2001).

Several studies have indicated a role for the Ami-AliA/AliB permease, encoded by the *amiACDEF* operon and the *aliA* and *aliB* genes, in pneumococcal virulence. Mutants in *amiA* and *amiC* displayed diminished adherence to pulmonary epithelial cells *in vitro* (Cundell *et al.*, 1995), and *aliB* and *amiACD* were identified in STM screens of pneumococcal pneumonia in a serotype 3 and 4 background, respectively (Lau *et al.*, 2001; Hava and Camilli, 2002). Furthermore, using a collection of *aliA*, *aliB* and *amiA* single or triple mutants, the Ami-AliA/AliB permease was shown to be required for successful nasopharyngeal colonization, but not for pneumococcal pneumonia, with the most pronounced phenotype for the triple and the *aliA* and *amiA* single mutants (Kerr *et al.*, 2004).

Inactivation of *codY* was previously reported to result in reduced adherence to nasopharyngeal cells and reduced colonization in a mouse model of pneumococcal infection (Hendriksen *et al.*, 2008). However, no significant differences in bacterial loads between wild type and the *codY* mutant were observed in pneumonia and bacteremia models of infection. In light of the above, we consider it unlikely that the truncation of *fatC* contributed to the virulence phenotype associated with *codY* inactivation. On the other hand, mutation of *amiC* may, at least partially, have been responsible for the observed attenuation during colonization, but further experiments are needed to exactly determine the impact of the *amiC* mutation on the virulence phenotype of the *codY* mutant.

Why is CodY essential in S. pneumoniae?

560 The finding that CodY is essential suggests that this regulator controls genes/functions that
561 are crucial for *S. pneumoniae*. The demonstration that mutations in *fatC* and *amiC* fully
562 restore viability of *codY* mutant cells may provide some clue as to these functions. As the
563 genome sequence analysis of D39 Δ *codY* strongly suggested that the *fatC* mutation arose first
564 during establishment of the *codY* mutant construct, it is tempting to speculate about a possible
565 toxicity of iron in cells lacking CodY. If CodY is a repressor of the *fatD-fatC-fecE-fatB*
566 operon as previously concluded (Hendriksen *et al.*, 2008), derepression of iron uptake in its
567 absence may lead to accumulation of toxic concentrations of iron within the cell, thus creating
568 a strong selection pressure for the emergence of *fat* mutants. In this context, what could
569 account for the concomitant accumulation of *amiC* mutations? Considering only Ami and
570 CodY, it could be speculated that *codY* inactivation leading to derepression of the many
571 transporters of aa controlled by CodY and of Ami results in lethal imbalance of aminoacid
572 pools. However, this explanation is not readily connected to the co-occurrence of *fatC*
573 inactivation. In search of a possible direct connection, we came across the observation that the
574 housekeeping dipeptide permease of *Escherichia coli* allows utilization of heme as an iron
575 source (Letoffe *et al.*, 2006). Heme utilization has thus been shown to require a permease
576 made up of DppBCDF (dipeptide inner membrane transporter) as the ABC transporter and
577 either MppA (periplasmic L-alanyl- γ -D-glutamyl-meso-diaminopimelate binding protein) or
578 DppA (periplasmic dipeptide binding protein) as the substrate-binding protein. As *S.*
579 *pneumoniae* is devoid of a Dpp system, it is tempting to speculate that the Ami-Obl
580 oligopeptide transporter homologous to Dpp could allow heme utilization as an iron source.
581 The simultaneous involvement of CodY in *ami-obl* and *fat-fec* repression, and in *dpr*
582 activation would, upon *codY* inactivation, generate a severe oxidative stress because of the
583 simultaneous derepression of iron uptake via two transporters and depletion of the iron
584 storage-peroxide resistance Dpr protein (Fig. 7), hence the observed accumulation of *fat* and

585 *ami* mutations in the $\Delta codY$ background. It may not be a mere coincidence that the *ami* and *fat*
586 mutations which restore viability of *codY* mutant cells lead to competence (X-state)
587 derepression (Fig. 5 and Fig. S5). Since X-state is considered a pneumococcal substitute for
588 SOS (Claverys *et al.*, 2006; Prudhomme *et al.*, 2006), its induction may counteract oxidative
589 stress in *codY* mutant cells. Further investigation should reveal whether induction of the X-
590 state is required for tolerance of *codY* inactivation.

591 Transcriptome analysis revealed a striking parallel between CodY and RitR, an orphan
592 two-component signal transduction response regulator. Increased transcription of *piuB* and
593 *piuA* (i.e. *fatD* and *fatB*), as well as of *amiC* and decreased expression of *dpr* were reported in
594 *ritR* mutant cells (Ulijasz *et al.*, 2004) suggesting that CodY and RitR share these targets (Fig.
595 7). RitR was shown to bind three sites (RRB1-3; Fig. 6A) in the promoter region of the *fat*
596 operon (Ulijasz *et al.*, 2004). Recently, regulation of RitR binding at the *fat* promoter by a
597 Ser-Thr kinase-phosphatase, StkP-PhpP, was documented (Ulijasz *et al.*, 2009). While RitR
598 and PhpP jointly participate in complex formation at the *fat* promoter *in vitro*, addition of
599 StkP was shown to disrupt the complexes (Ulijasz *et al.*, 2009). This finding was consistent
600 with DNA microarray analyses of transcripts from an *stkP* knock-out showing dependence of
601 *fat* expression on StkP (Saskova *et al.*, 2007). The overlap between the CodY box
602 (AATTGTCAGAAATT located 3 nucleotides upstream of the -35 promoter box) and the first
603 RitR box (Fig. 6A) suggests that CodY may also interfere with RitR binding and adds a
604 degree to the complexity of *fat* regulation. CodY may thus represent one of the additional *fat*
605 regulators, the existence of which was proposed to account for the failure to detect the
606 opposite effects predicted for the individual deletion of *phpP* and *stkP* (Ulijasz *et al.*, 2009).
607 In line with the oxidative stress hypothesis (Fig. 7), *ritR* mutant cells were shown to display
608 greatly increased susceptibility to streptonigrin, which requires the presence of intracellular
609 iron, as well as to hydrogen peroxide (Ulijasz *et al.*, 2004). Iron overload due to derepression

of *fat* could be responsible for the latter by increasing intracellular free iron concentration and therefore the potential for synthesis of reactive oxygen intermediates. If both CodY and RitR are important for iron homeostasis and the reason for CodY essentiality is to prevent oxidative stress, what about the viability of *ritR* mutant cells? As there was no mention of difficulty in generating and/or growing *ritR* mutants, CodY might be more important for *ami*, *fat* and *dpr* regulation than RitR. Alternatively, RitR and CodY could be equally important and the problematic viability of *ritR* mutant cells has been overlooked. It would therefore be interesting to check whether a previously constructed *ritR* mutant is readily transferred by transformation.

Concluding remarks

Further work with other clinical isolates of *S. pneumoniae* is necessary to establish whether the essentiality of CodY is a general feature of this species. To the best of our knowledge, this is the first report in any bacterium that a member of the CodY family is essential. Is this situation unique to *S. pneumoniae*? It would be interesting to investigate whether CodY is essential in species closely related to *S. pneumoniae* and in streptococci in general. It is possible that CodY is also essential in other species but that the presence of suppressor mutation(s) has been overlooked. Our observations may thus prompt careful reexamination of the viability of the *codY* mutants previously constructed in other species. More generally, the above described tests of acceptance frequencies by transformation should be used routinely when working with 'important' genes, to prevent the presence of suppressors going undetected in the future. In any case, because of its essentiality in a major human pathogen, CodY constitutes a potentially interesting new therapeutic target.

Experimental procedures

Bacterial strains, culture and transformation conditions

S. pneumoniae strains and plasmids used in this study are described in Table 1. Stock cultures were routinely grown at 37°C in Todd-Hewitt plus yeast extract (THY) medium to OD₅₅₀=0.3; after addition of 15% glycerol, stocks were kept frozen at -70°C. To investigate spontaneous competence development, cells were gently thawed and aliquots were inoculated (1 into 25) in C+Y. The initial pH value was adjusted to 7.0 and trypsin (2 µg ml⁻¹) was added to prevent spontaneous competence induction in the preculture. After incubation at 37°C to OD₅₅₀=0.2, cultures were centrifuged and cells were concentrated to OD₅₅₀ of 0.4 in fresh medium containing 15% glycerol and kept frozen at -70°C. For the monitoring of growth and spontaneous competence development, these precultures were gently thawed and aliquots were inoculated (1 into 50, unless otherwise indicated) in luciferin-containing C+Y medium and distributed into a 96-well microplate (300 µl per well). Measurement of competence involved the use of an *ssbB::luc* transcriptional fusion which reports on competence through light emission by luciferase (Prudhomme and Claverys, 2007). RLU (relative luminescence unit) and OD values were recorded throughout incubation at 37°C (in a Varioskan Flash luminometer; Thermo Electron Corporation).

CSP-induced transformation was performed as described previously (Martin *et al.*, 2000), using precompetent cells treated at 37°C for 10 min with synthetic CSP1 (100 ng ml⁻¹). After addition of transforming DNA, cells were incubated for 20 minutes at 30°C. Transformants were selected by plating on CAT-agar supplemented with 4% horse blood, followed by

challenge with a 10 ml overlay containing chloramphenicol ($4.5 \mu\text{g ml}^{-1}$), erythromycin ($0.05 \mu\text{g ml}^{-1}$), kanamycin ($250 \mu\text{g ml}^{-1}$), methotrexate ($2.2 \mu\text{g ml}^{-1}$), spectinomycin ($100 \mu\text{g ml}^{-1}$), streptomycin ($200 \mu\text{g ml}^{-1}$) or trimethoprim ($20 \mu\text{g ml}^{-1}$), after phenotypic expression for 120 min at 37°C .

Mutagenesis and duplication of codY

Insertions of *kan* (Kan^R) or *spc* (Spc^R) minitransposons were generated by *in vitro mariner* mutagenesis as described (Prudhomme *et al.*, 2007). Plasmids used as a source for the minitransposons were pR410 and pR412, respectively (Table 1). Briefly, plasmid DNA ($\sim 1 \mu\text{g}$) was incubated with a target PCR fragment (indicated in the legend of Fig. 1) in the presence of purified *HimarI* transposase, leading to random insertion of the minitransposon within the fragment. Gaps in transposition products were repaired as described (Prudhomme *et al.*, 2007) and the resulting *in vitro*-generated transposon insertion library was used to transform *S. pneumoniae*. Location and orientation of minitransposon insertions were determined as previously described (Prudhomme *et al.*, 2007) through PCR reactions using primers MP127 or MP128 in combination with either one of the primers used to generate *codY* PCR fragments (Table 1). Cassette-chromosome junctions were sequenced for some insertions as indicated in the legend of Fig. 1.

Placement of a second copy of *codY* under the control of the maltose-inducible P_M promoter at CEP was achieved by cloning into *NcoI*-*Bam*HI digested pCEP2 plasmid DNA a *codY* PCR fragment generated using the *codY*atg-*codY*stop primer pair (Table 1) and digested with *Bam*HI and *Nde*I. The resulting recombinant plasmid pCEP-*codY* was used as donor in transformation of strain R1501 followed by selection for a Kan^R transformant, thus generating

strain R2349 (Table 1). Plasmid pCEP2 was generated in this study as a high copy number derivative of plasmid pCEP (Guiral *et al.*, 2006). Briefly, an *EcoRI-PstI* fragment from pCEP was ligated to *EcoRI-PstI* digested pKL147 (Table 1) to replace the pSC101 replication machinery of pCEP by the pBR replication machinery and Ap^R resistance gene of pKL147.

Reversion of *codY* knock-out by transformation

To replace *codY::trim* or *codY::spc* insertions by *codY*⁺, we took advantage of the *kan*^{90C} *mariner* insertion. This insertion is located immediately upstream the CodY binding site (CYB) in the *codY* promoter region (Fig. 1A) and does not inactivate *codY*; we used it as a marker to select for the re-introduction of the *codY*⁺ gene by co-transformation with Kan^R.

To allow the use of a similar strategy for replacement of *codY::trim* in *kan*^R strains (such as TD83), the *kan*^{90C} cassette was exchanged with the *spc*^R cassette by transformation of strain R2641 with plasmid pR412 DNA, selecting for Spc^R transformants to generate strain R2644 (genotype referred to as *spc*^{90C}::*codY*⁺; Table 1). The exchange is based on the presence of DNA homology at the borders of the synthetic *spc* and *kan* minitransposons, allowing exchange of the resistance cassette genes by homologous recombination during transformation.

Whole genome sequencing of D39 and D39Δ*codY*

Roche 454 FLX whole genome sequencing was performed by Agowa Genomics (Berlin, Germany) using genomic DNA isolated from mid-log cultures by the Genomic DNA kit

(Qiagen). For each strain, a shotgun library and a 3-kb span paired end library were generated according to Roche standard protocols, mixed in equal parts (about 400,000 beads from each library) and sequenced using default settings on a ¼ picotiterplate. A total of 240496 reads of which 65336 contained paired ends were obtained for D39 Δ *codY* (29-fold coverage), and 210631 reads with 60676 paired ends were obtained for D39 (25-fold coverage). *De novo* assembly was carried out using the Roche 454 Newbler software (Release 2.3 (091027_1459)), resulting in 68 contigs in 5 scaffolds for D39 Δ *codY*, and 85 contigs in 5 scaffolds for D39.

Data from the sequencing runs were mapped to the reference D39 strain (Acc.no.: NC_008533.1) and the variations thereto scored using the Roche 454 Reference Mapper software [Release 2.3 (091027_1459)].

Sequencing of amiC and fatC of individual D39 Δ codY clones

The *amiC* and *fatC* sequence of the D39 Δ *codY* population was verified by a PCR-sequencing approach. To this end, chromosomal DNA was isolated from individual clones by cetyltrimethylammonium bromide (CTAB) extraction as described previously (van Soolingen *et al.*, 1994). The *amiC* and *fatC* loci were PCR-amplified under standard conditions using, respectively, primers HBDamiCF1 and HBDamiCR2 and HBDfatCF and HBDfatCR (Table 1). Subsequently, both strands were sequenced using the same primers used for PCR as well as internal primers HBDamiCR1 and HBDamiCF2 (Table 1) in case of *amiC*.

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Supplementary material

The following Supplementary Material is available for this article:

Fig. S1. Attempt at depleting CodY and consequences on growth and competence in C+Y medium.

Table S1. Variations between the D39 NCTC 7466 isolate sequenced in this study and the previously published D39 NCTC 7466 genome sequence (Lanè *et al.*, 2007).

Fig. S2. Proposed unique mutational event responsible for the *amiC*^{G246TT} *amiC*^{A248C} (or *amiC*^{GGA→TTGC}) changes.

Fig. S3. Localization of *ami* mutation in strain D39Δ*cps*Δ*codY* via transformation.

Table S2. The original D39Δ*codY* strain displays a mixed population structure with respect to *amiC*.

Fig. S4. Distribution of *spc mariner* minitransposon insertions in the *fat-fec* operon.

Fig. S5. Effect of *ami*, *fat* and *ami fat* inactivation on growth and spontaneous competence development.

References

- Akerley, B.J., Rubin, E.J., Camilli, A., Lampe, D.J., Robertson, H.M., and Mekalanos, J.J. (1998) Systematic identification of essential genes by *in vitro* mariner mutagenesis. *Proc Natl Acad Sci USA* **95**: 8927-8932.
- Alloing, G., de Philip, P., and Claverys, J.P. (1994) Three highly homologous membrane-bound lipoproteins participate in oligopeptide transport by the Ami system of the Gram-positive *Streptococcus pneumoniae*. *J Mol Biol* **241**: 44-58.
- Alloing, G., Martin, B., Granadel, C., and Claverys, J.P. (1998) Development of competence in *Streptococcus pneumoniae*: pheromone auto-induction and control of quorum-sensing by the oligopeptide permease. *Mol Microbiol* **29**: 75-84.
- Attaiech, L., Granadel, C., Claverys, J.P., and Martin, B. (2008) RadC, a misleading name? *J Bacteriol* **190**: 5729-5732.
- Bergé, M., Moscoso, M., Prudhomme, M., Martin, B., and Claverys, J.P. (2002) Uptake of transforming DNA in Gram-positive bacteria: a view from *Streptococcus pneumoniae*. *Mol Microbiol* **45**: 411-421.
- Bootsma, H.J., Egmont-Petersen, M., and Hermans, P.W. (2007) Analysis of the *in vitro* transcriptional response of human pharyngeal epithelial cells to adherent *Streptococcus pneumoniae*: evidence for a distinct response to encapsulated strains. *Infect Immun* **75**: 5489-5499.
- Brown, J.S., Gilliland, S.M., and Holden, D.W. (2001) A *Streptococcus pneumoniae* pathogenicity island encoding an ABC transporter involved in iron uptake and virulence. *Mol Microbiol* **40**: 572-585.
- Brown, J.S., Gilliland, S.M., Ruiz-Albert, J., and Holden, D.W. (2002) Characterization of pit, a *Streptococcus pneumoniae* iron uptake ABC transporter. *Infect Immun* **70**: 4389-4398.
- Burghout, P., Bootsma, H.J., Kloosterman, T.G., Bijlsma, J.J., de Jongh, C.E., Kuipers, O.P., and Hermans, P.W. (2007) Search for genes essential for pneumococcal transformation: the RADA DNA repair protein plays a role in genomic recombination of donor DNA. *J Bacteriol* **189**: 6540-6550.
- Chastanet, A., Prudhomme, M., Claverys, J.P., and Msadek, T. (2001) Regulation of *Streptococcus pneumoniae* *clp* genes and their role in competence development and stress survival. *J Bacteriol* **183**: 7295-7307.
- Chen, J.D., and Morrison, D.A. (1987) Modulation of competence for genetic transformation in *Streptococcus pneumoniae*. *J Gen Microbiol* **133**: 1959-1967.

- 801 Claverys, J.P., Grossiord, B., and Alloing, G. (2000) Is the Ami-AliAB oligopeptide permease
802 of *Streptococcus pneumoniae* involved in sensing environmental conditions? *Res Microbiol*
803 **151**: 1-7.
- 804 Claverys, J.P., and Håvarstein, L.S. (2002) Extra-cellular peptide control of competence for
805 genetic transformation in *Streptococcus pneumoniae*. *Frontiers in Biosci* **7**: 1798-1814.
- 806 Claverys, J.P., and Lacks, S.A. (1986) Heteroduplex deoxyribonucleic acid base mismatch
807 repair in bacteria. *Microbiol Rev* **50**: 133-165.
- 808 Claverys, J.P., Prudhomme, M., and Martin, B. (2006) Induction of competence regulons as
809 general stress responses in Gram-positive bacteria. *Annu Rev Microbiol* **60**: 451-475.
- 810 Cundell, D.R., Pearce, B.J., Sandros, J., Naughton, A.M., and Masure, H.R. (1995) Peptide
811 permeases from *Streptococcus pneumoniae* affect adherence to eucaryotic cells. *Infect Immun*
812 **63**: 2493-2498.
- 813 Dagkessamanskaia, A., Moscoso, M., Hénard, V., Guiral, S., Overweg, K., Reuter, M. *et al.*
814 (2004) Interconnection of competence, stress and CiaR regulons in *Streptococcus*
815 *pneumoniae*: competence triggers stationary phase autolysis of *ciaR* mutant cells. *Mol*
816 *Microbiol* **51**: 1071-1086.
- 817 Gasc, A.M., Sicard, A.M., and Claverys, J.P. (1989) Repair of single- and multiple-
818 substitution mismatches during recombination in *Streptococcus pneumoniae*. *Genetics* **121**:
819 29-36.
- 820 Guédon, E., Serror, P., Ehrlich, S.D., Renault, P., and Delorme, C. (2001) Pleiotropic
821 transcriptional regulator CodY senses the intracellular pool of branched-chain amino acids in
822 *Lactococcus lactis*. *Mol Microbiol* **40**: 1227-1239.
- 823 Guiral, S., Hénard, V., Laaberki, M.-H., Granadel, C., Prudhomme, M., Martin, B., and
824 Claverys, J.P. (2006) Construction and evaluation of a chromosomal expression platform
825 (CEP) for ectopic, maltose-driven gene expression in *Streptococcus pneumoniae*.
826 *Microbiology (Special Issue on Pneumococcus)* **152**: 343-349.
- 827 Guiral, S., Mitchell, T.J., Martin, B., and Claverys, J.P. (2005) Competence-programmed
828 predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic
829 requirements. *Proc Natl Acad Sci USA* **102**: 8710-8715.
- 830 Handke, L.D., Shivers, R.P., and Sonenshein, A.L. (2008) Interaction of *Bacillus subtilis*
831 CodY with GTP. *J Bacteriol* **190**: 798-806.
- 832 Hava, D.L., and Camilli, A. (2002) Large-scale identification of serotype 4 *Streptococcus*
833 *pneumoniae* virulence factors. *Mol Microbiol* **45**: 1389-1406.
- 834 Håvarstein, L.S., Martin, B., Johnsborg, O., Granadel, C., and Claverys, J.P. (2006) New
835 insights into pneumococcal fratricide: relationship to clumping and identification of a novel
836 immunity factor. *Mol Microbiol* **59**: 1297-1307.
- 837 Hendriksen, W.T., Bootsma, H.J., Estevão, S., Hoogenboezem, T., De Jong, A., De Groot, R.
838 *et al.* (2008) CodY of *Streptococcus pneumoniae*: link between nutritional gene regulation
839 and virulence. *J Bacteriol* **190**: 590-601.

- 840 Johnsborg, O., and Havarstein, L.S. (2009) Pneumococcal LytR, a protein from the LytR-
841 CpsA-Psr family, is essential for normal septum formation in *Streptococcus pneumoniae*. *J*
842 *Bacteriol* **191**: 5859-5864.
- 843 Kerr, A.R., Adrian, P.V., Estevão, S., De Groot, R., Alloing, G., Claverys, J.P. *et al.* (2004)
844 The Ami-AliA/B permease of *Streptococcus pneumoniae* is involved in nasopharyngeal
845 colonization but not in invasive disease. *Infect Immun* **72**: 3902-3906.
- 846 Kloosterman, T.G., Hendriksen, W.T., Bijlsma, J.J., Bootsma, H.J., van Hijum, S.A., Kok, J.
847 *et al.* (2006) Regulation of glutamine and glutamate metabolism by GlnR and GlnA in
848 *Streptococcus pneumoniae*. *J Biol Chem* **281**: 25097-25109.
- 849 Lanie, J.A., Ng, W.L., Kazmierczak, K.M., Andrzejewski, T.M., Davidsen, T.M., Wayne, K.J.
850 *et al.* (2007) Genome Sequence of Avery's Virulent Serotype 2 Strain D39 of *Streptococcus*
851 *pneumoniae* and Comparison with That of Unencapsulated Laboratory Strain R6. *J Bacteriol*
852 **189**: 38-51.
- 853 Lau, G.W., Haataja, S., Lonetto, M., Kensit, S.E., Marra, A., Bryant, A.P. *et al.* (2001) A
854 functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. *Mol Microbiol*
855 **40**: 555-571.
- 856 Lefèvre, J.C., Claverys, J.P., and Sicard, A.M. (1979) Donor deoxyribonucleic acid length and
857 marker effect in pneumococcal transformation. *J Bacteriol* **138**: 80-86.
- 858 Lemon, K.P., and Grossman, A.D. (1998) Localization of Bacterial DNA Polymerase:
859 Evidence for a Factory Model of Replication. *Science* **282**: 1516-1519.
- 860 Letoffe, S., Delepelaire, P., and Wandersman, C. (2006) The housekeeping dipeptide
861 permease is the *Escherichia coli* heme transporter and functions with two optional peptide
862 binding proteins. *Proc Natl Acad Sci U S A* **103**: 12891-12896.
- 863 Martin, B., Prudhomme, M., Alloing, G., Granadel, C., and Claverys, J.P. (2000) Cross-
864 regulation of competence pheromone production and export in the early control of
865 transformation in *Streptococcus pneumoniae*. *Mol Microbiol* **38**: 867-878.
- 866 Molle, V., Nakaura, Y., Shivers, R.P., Yamaguchi, H., Losick, R., Fujita, Y., and Sonenshein,
867 A.L. (2003) Additional targets of the *Bacillus subtilis* global regulator CodY identified by
868 chromatin immunoprecipitation and genome-wide transcript analysis. *J Bacteriol* **185**: 1911-
869 1922.
- 870 Mortier-Barrière, I., de Saizieu, A., Claverys, J.P., and Martin, B. (1998) Competence-specific
871 induction of *recA* is required for full recombination proficiency during transformation in
872 *Streptococcus pneumoniae*. *Mol Microbiol* **27**: 159-170.
- 873 Pericone, C.D., Park, S., Imlay, J.A., and Weiser, J.N. (2003) Factors contributing to
874 hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB)
875 and avoidance of the toxic effects of the fenton reaction. *J Bacteriol* **185**: 6815-6825.
- 876 Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B., and Claverys, J.P. (2006) Antibiotic
877 stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*.
878 *Science* **313**: 89-92.

- 879 Prudhomme, M., Camilli, A., and Claverys, J.P. (2007) *In vitro mariner* mutagenesis of
880 *Streptococcus pneumoniae*: tools and traps. In *The Molecular Biology of Streptococci*.
881 Hakenbeck, R., and Chhatwal, G.S. (eds). Horizon Scientific Press, pp. 511-518.
- 882 Prudhomme, M., and Claverys, J.P. (2007) There will be a light: the use of *luc* transcriptional
883 fusions in living pneumococcal cells. In *The Molecular Biology of Streptococci*. Hakenbeck,
884 R., and Chhatwal, G.S. (eds). Horizon Scientific Press, pp. 519-524.
- 885 Puyet, A., Ibáñez, A.M., and Espinosa, M. (1993) Characterization of the *Streptococcus*
886 *pneumoniae* maltosaccharide regulator MalR, a member of the LacI-GalR family of repressors
887 displaying distinctive genetic features. *J Biol Chem* **268**: 25402-25408.
- 888 Ratnayake-Lecamwasam, M., Serror, P., Wong, K.W., and Sonenshein, A.L. (2001) *Bacillus*
889 *subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. *Genes Dev* **15**:
890 1093-1103.
- 891 Saskova, L., Novakova, L., Basler, M., and Branny, P. (2007) Eukaryotic-type
892 serine/threonine protein kinase StkP is a global regulator of gene expression in *Streptococcus*
893 *pneumoniae*. *J Bacteriol* **189**: 4168-4179.
- 894 Serror, P., and Sonenshein, A.L. (1996) CodY is required for nutritional repression of *Bacillus*
895 *subtilis* genetic competence. *J Bacteriol* **178**: 5910-5915.
- 896 Sonenshein, A.L. (2005) CodY, a global regulator of stationary phase and virulence in Gram-
897 positive bacteria. *Curr Opin Microbiol* **8**: 203-207.
- 898 Sonenshein, A.L. (2007) Control of key metabolic intersections in *Bacillus subtilis*. *Nat Rev*
899 *Microbiol* **5**: 917-927.
- 900 Sung, C.K., Li, H., Claverys, J.P., and Morrison, D.A. (2001) An *rpsL* Cassette, Janus, for
901 Gene Replacement through Negative Selection in *Streptococcus pneumoniae*. *Appl Environ*
902 *Microbiol* **67**: 5190-5196.
- 903 Tai, S.S., Yu, C., and Lee, J.K. (2003) A solute binding protein of *Streptococcus pneumoniae*
904 iron transport. *FEMS Microbiol Lett* **220**: 303-308.
- 905 Thanassi, J.A., Hartman-Neumann, S.L., Dougherty, T.J., Dougherty, B.A., and Pucci, M.J.
906 (2002) Identification of 113 conserved essential genes using a high-throughput gene
907 disruption system in *Streptococcus pneumoniae*. *Nucleic Acids Res* **30**: 3152-3162.
- 908 Tiraby, G., Fox, M.S., and Bernheimer, H. (1975) Marker discrimination in deoxyribonucleic
909 acid-mediated transformation of various pneumococcus strains. *J Bacteriol* **121**: 608-618.
- 910 Ulijasz, A.T., Andes, D.R., Glasner, J.D., and Weisblum, B. (2004) Regulation of iron
911 transport in *Streptococcus pneumoniae* by RitR, an orphan response regulator. *J Bacteriol*
912 **186**: 8123-8136.
- 913 Ulijasz, A.T., Falk, S.P., and Weisblum, B. (2009) Phosphorylation of the RitR DNA-binding
914 domain by a Ser-Thr phosphokinase: implications for global gene regulation in the
915 streptococci. *Mol Microbiol* **71**: 382-390.

- 916 van Soolingen, D., de Haas, P.E., Hermans, P.W., and van Embden, J.D. (1994) DNA
917 fingerprinting of *Mycobacterium tuberculosis*. *Methods Enzymol* **235**: 196-205.

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Table 1. Strains, plasmids, and primers used in this study.

Strains	Genotype ^a /description	Source/reference
<i>Streptococcus pneumoniae</i>		
D39	Serotype 2	NCTC 7466
D39 Δ <i>codY</i>	D39 Δ <i>codY::trim (socY*)</i> ^b ; Trim ^R , (Mtx ^R) ^b	(Hendriksen <i>et al.</i> , 2008)
D39 Δ <i>cps</i>	D39 Δ <i>cps::kan</i> ; Kan ^R	(Hendriksen <i>et al.</i> , 2008)
D39 Δ <i>cps</i> Δ <i>codY</i>	D39 Δ <i>cps::kan</i> Δ <i>codY::trim (socY)</i> ^c ; Kan ^R , Trim ^R , (Mtx ^R) ^c	(Hendriksen <i>et al.</i> , 2008)
R6	Unencapsulated derivative of D39	(Burghout <i>et al.</i> , 2007)
R246	R800 but <i>hexA</i> Δ 3:: <i>ermAM</i> ; Ery ^R	(Mortier-Barrière <i>et al.</i> , 1998)
R304	R800 derivative, <i>nov1</i> , <i>rif23</i> , <i>str41</i> ; Nov ^R , Rif ^R , Sm ^R	(Mortier-Barrière <i>et al.</i> , 1998)
R800	R6 derivative	(Lefèvre <i>et al.</i> , 1979)
R895	R800 but <i>ssbB::luc (ssbB⁺)</i> ; Cm ^R	(Chastanet <i>et al.</i> , 2001)
R1501	R800 but <i>comC</i> ₀	(Dagkessamanskaia <i>et al.</i> , 2004)
R1818	R1501 but <i>hexA</i> Δ 3:: <i>ermAM</i> ; Ery ^R	This study
R2349	R1501 but CEP _M - <i>codY</i> (the resulting duplication of <i>codY</i> is denoted <i>codY</i> ^{+/+}); Kan ^R	This study
R2350	R895 but <i>codY</i> ^{+/+} ; Kan ^R , Cm ^R	This study
R2424	R2350 but genuine <i>codY</i> inactivated by <i>mariner</i> insertion <i>spc</i> ^{3A} (the resulting <i>codY</i> combination is denoted <i>codY</i> ^{spc3/+}); Kan ^R , Cm ^R , Spc ^R	This study

R2425	R2350 but ectopic <i>codY</i> inactivated by <i>mariner</i> insertion <i>spc</i> ^{3A} (the resulting <i>codY</i> combination is denoted <i>codY</i> ^{+/spc3}); Kan ^R , Cm ^R , Spc ^R	This study
R2427	R6 but <i>codY</i> ^{+/+} ; Kan ^R	This study
R2428	R1818 but <i>codY</i> ^{+/+} ; Ery ^R , Kan ^R	This study
R2430	R895 but <i>pmalR</i> (pAPM22); Cm ^R , Ery ^R	This study
R2432	R2424 but <i>pmalR</i> (pAPM22) ; Cm ^R , Ery ^R , Kan ^R , Spc ^R	This study
R2437	R2349 but <i>str41</i> ; Kan ^R , Str ^R	This study
R2438	R2437 but <i>codY</i> ^{spc3/+} ; Kan ^R , Spc ^R , Str ^R	This study
R2549	R895 but <i>amiC9</i> ; Cm ^R , Mtx ^R	This study
R2641	R895 but <i>kan</i> ^{90C} :: <i>codY</i> ⁺ , <i>str41</i> ; Cm ^R , Spc ^R , Str ^R	This study
R2644	R2641 but <i>spc</i> ^{90C} :: <i>codY</i> ⁺ ; Cm ^R , Spc ^R , Str ^R	This study
R2737	R895 but CEP _M ; Cm ^R , Kan ^R	This study
R3002	R246 but <i>fatC</i> :: <i>cat</i> ^{23C} (from TD131); Cm ^R , Ery ^R	This study
R3003	R246 but <i>amiC9</i> ; Ery ^R , Mtx ^R	This study
R3004	R3002 but <i>amiC9</i> ; Cm ^R , Ery ^R , Mtx ^R	This study
TD73	D39 but <i>codY</i> ^{+/+} ; Kan ^R	This study
TD80	D39Δ <i>cps</i> Δ <i>codY</i> (<i>socY</i>) ^b but <i>str41</i> ; Kan ^R , Str ^R , Trim ^R	This study
TD81	TD80 but <i>codY</i> :: <i>spc</i> ^{3A} ; Kan ^R , Spc ^R , Str ^R	This study
TD82	D39Δ <i>cps</i> but <i>ssbB</i> :: <i>luc</i> (<i>ssbB</i> ⁺); Cm ^R , Kan ^R	This study
TD83	D39Δ <i>cps</i> Δ <i>codY</i> but <i>ssbB</i> :: <i>luc</i> (<i>ssbB</i> ⁺); Cm ^R , Kan ^R , Trim ^R	This study
TD84	TD80 but <i>amiC9</i> ; Cm ^R , Kan ^R , Mtx ^R	This study
TD95	TD83 but <i>spc</i> ^{90C} :: <i>codY</i> ⁺ ; Cm ^R , Kan ^R , Spc ^R	This study
TD96	TD95 but <i>codY</i> ⁺ ; Cm ^R , Kan ^R	This study
TD129	D39Δ <i>cps</i> but <i>fatC</i> :: <i>spc</i> ^{23C} ; Kan ^R , Spc ^R	This study
TD130	TD129 but <i>amiC9</i> ; Kan ^R , Mtx ^R , Spc ^R	This study
TD131	TD129 but <i>fatC</i> :: <i>cat</i> ^{23C} ; Cm ^R , Kan ^R	This study
TD135	TD130 but <i>ssbB</i> :: <i>luc</i> (<i>ssbB</i> ⁺); Cm ^R , Kan ^R , Mtx ^R , Spc ^R	This study

TD138	TD129 but <i>ssbB::luc</i> (<i>ssbB</i> ⁺); Cm ^R , Kan ^R , Spc ^R	This study
TD154	TD135 but $\Delta codY::trim$; Cm ^R , Kan ^R , Mtx ^R , Spc ^R , Trim ^R	This study
Plasmids		
pAPM22	pLS1 derivative carrying the <i>malR</i> gene; Ery ^R	(Puyet <i>et al.</i> , 1993)
pCEP	pSC101 derivative (i.e. low copy number plasmid) carrying CEP; Spc ^R , Kan ^R	(Guiral <i>et al.</i> , 2006)
pCEP2	pKL147 derivative (i.e. high copy number plasmid) carrying an <i>EcoRI/PstI</i> fragment from pCEP; Spc ^R , Kan ^R , Ap ^R	This study
pCEP2- <i>codY</i>	pCEP2 derivative carrying <i>codY</i> under P _M control; Kan ^R , Ap ^R	Spc ^R , This study
pEMcat	ColE1 derivative carrying a Cm ^R <i>mariner</i> minitransposon; Ap ^R , Cm ^R	(Akerley <i>et al.</i> , 1998)
pKL147	pUS19 derivative containing <i>gfpmut2</i> fused to the 3' end of <i>dnaX</i> with a linker; Spc ^R , Ap ^R	(Lemon and Grossman, 1998)
pR410	pEMcat derivative carrying a Kan ^R (<i>kan</i> gene) <i>mariner</i> minitransposon; Ap ^R , Kan ^R	(Sung <i>et al.</i> , 2001)
pR412	pEMcat derivative carrying a Spc ^R (<i>aad9</i> gene, also called <i>spc</i>) <i>mariner</i> minitransposon; Ap ^R , Spc ^R	(Martin <i>et al.</i> , 2000)
Primers		
	Sequence^d; gene; position[§]	
ami1	GCGCAAACAGGCTCTAAGGG; <i>amiA</i> ; +1815	This study
ami2	TCAGGAATTCCTGCTGCCATTAT; <i>amiC</i> ; +1257	This study
ami4	CCTGACTCACCTACCAAGGCTA; <i>amiD</i> ; +712	This study
ami5	CCTTCACCGAAGGAAATTTCTA; <i>amiE</i> ; +121	This study
ami6	TTAGCTGACTTCAACCCACTACA; <i>amiF</i> ; +1027	This study
amiF1	GCCTTGCTTTTCAGCGGTACCAAT; <i>amiF</i> ; +789	This study

AM40	AGAGTTTCGGATGGTTTGGA; <i>treR</i> ; +347	This study
codY1	CAAGGATCAGTTTTCCCATATTTTCG; <i>codY</i> ; +1636	This study
codY2	CTTCGTGTCCTTCGTGACTTTA; <i>codY</i> ; -1004	This study
codYatg	tgaatc ATG aCACATTTATTAGAAAAAACTAG; <i>codY</i> ; 0	This study
codYstop	aaattgga tc TTTGTCAT T AGTAATCTCTTTTC; <i>codY</i> ; +797	This study
fat1	GCGAACGAATGATTTACTGG; <i>fatD</i> ; -659	This study
fat2	TCTCACCAGTCTTTCCACCC; <i>fatB</i> ; +1388	This study
fatC1	TAAAAGCAAACATACCAAGC; <i>fatC</i> ; -9	This study
fatC2	TAAAGAATAAGAAGCCACCC; <i>fatC</i> ; +909	This study
HBDamiCF1	ACGGCTGATAAACGTGATAA; <i>amiC</i> ; +145	This study
HBDamiCF2	GTCGTTGGTCTTGTCTTCAT; <i>amiC</i> ; +1381	This study
HBDamiCR1	TAAATTCTCCCAAAGTCCAA; <i>amiC</i> ; +343	This study
HBDamiCR2	CGCATCAATAGTTTCAGAGG; <i>amiC</i> ; +1568	This study
HBDfatCF	ACACTGATGAAGCAAGACCT; <i>fatC</i> ; +376	This study
HBDfatCR	CAATATCTGAGCCGTTTCTC; <i>fatC</i> ; +645	This study
kan1	ATCATGTCCTTTTCCCGTTCCAC; <i>kan</i> ; +191	This study
MP127	CCGGGGACTTATCAGCCAACC; mariner transposon	(Martin <i>et al.</i> , 2000)
MP128	TACTAGCGACGCCATCTATGTG; mariner transposon	(Martin <i>et al.</i> , 2000)
MP188	TTCATTTTCACCAACCAGGTTAC; <i>codY</i> ; +1032	This study
MP189	ATTGGCTGCTGAGTTTACTCCAG; <i>codY</i> ; -618	This study

MP192	<u>ggatcc</u> ACGTCATCAACTAAATAGCG; <i>aliA</i> ; -343	This study
MP193	CAGAAGCTTTCTGGTTTGTGTT; <i>aliA</i> ; -539	This study
MP194	TTGGAATTCCCTCTTCTGGAAC; <i>dexB</i> ; +908	This study
MP195	ttagttgatgacgtg <u>gatcc</u> GCTTTTTATACAGTCCTCCC; <i>dexB</i> ; +1693	This study
rpsL_3	TGACATGGATACGGAAGTAG; <i>rspL</i> ; -798	This study
rpsL_4	ATGGTAAGCTGAGTTATAGC; <i>rpsL</i> ; +1204	This study

^a Ap^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Kan^R, kanamycin resistance; Mtx^R, methotrexate resistance; Spc^R, spectinomycin resistance; Str^R, streptomycin resistance; Trim^R, trimethoprim resistance.

^b *socY** refers to the presence of the *amiC*²³, *amiC*^{A248C} or *amiC*^{G1459T} mutations as well as the *fatC*^{C496T} identified in this study and demonstrated to be required for tolerance of *codY* inactivation.

^c *socY* refers to the presence of the *amiC*^{G1459T} and *fatC*^{C496T} mutations.

^d Lowercase letters indicate nucleotide extensions to introduce convenient restriction sites (*Nde*I, *Bam*HI, and *Eco*RI, respectively in *codY*atg, *codY*stop, and MP192; underlined sequences) in the primers [Note that the *Nde*I site in *codY*atg introduced a Ala → Thr change at the second aa position of CodY]. The start and stop codons of *codY* are shown in bold. In MP195, lowercase letters indicate nucleotide extension complementary to MP192.

937 §Position is given with respect to the ATG of the corresponding gene; - and + indicate
938 upstream and downstream, respectively.
939
940 ^C and ^A indicate respectively the co-transcribed and the reverse orientation of an inserted mini-
941 transposon antibiotic resistance cassette with respect to the targeted gene.

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942 **Fig. 1.** Insertion mutagenesis of *codY* is not possible in *S. pneumoniae* R6 unless the strain
943 carries an ectopic complementing copy of the gene.

944 A. Distribution of *mariner* insertions in the MP188-MP189 fragment obtained in wild type
945 cells.

946 B. Placement of a second copy of *codY* under the control of the maltose-inducible P_M
947 promoter at CEP.

948 The inset in (B) shows control PCRs with the primer pair amiF1-AM40: lane 1, 2083 bp-long
949 wildtype fragment; lane 2, 2760 bp-long CEP_M-*codY*-*kan* fragment. M, kb ladder.

950 C Recovery of *mariner* insertions at the *codY* locus in a recipient harboring a complementing
951 ectopic copy.

952 The location of primers used to generate *codY* PCR fragments for *mariner* mutagenesis,
953 MP188-MP189 (A) and codY1-codY2 (C) is indicated. Insertion sites were located through
954 DNA sequencing at position -100 for *kan*^{90C} (A) and -32, +163, +168, +300, and +365 for
955 *spc*^{5A}, *spc*^{4C}, *spc*^{3A}, *spc*^{12A}, and *spc*^{8A}, respectively (C). Positions are given with respect to the
956 first nucleotide of *codY* taken as +1; ^C and ^A indicate co-transcribed and reverse orientation of
957 *spc* (*s*) or *kan* (*k*) cassette, respectively, with respect to *codY*. A CodY box
958 (AATTTTCAGATAATT) previously identified in the promoter region of *codY* (Hendriksen
959 *et al.*, 2008) is indicated as CYB. It is separated by 1 nt from the putative -35 promoter box of
960 *codY*, itself separated by 17 nt from an extended -10 box with perfect match to the consensus
961 (last nucleotide of this -10 located 26 nt upstream of the *codY* start).

Fig. 2. Integration of *mariner* minitransposons can occur at either site in the chromosome of *codY*^{+/+} recipient cells.

A. PCR probing of the structure of an *Spc*^R transformant (strain R2424) carrying the *codY::spc*^{3A} minitransposon at the genuine *codY* locus using the following primer pairs: *codY*1-*codY*2 (fragment/lane 2, 3787 bp), *codY*2-MP127 (fragment/lane 4, 1493 bp), MP127-*codY*1 (fragment/lane 3, 1196 bp), and *amiF*1-AM40 (fragment/lane 5, 1684 bp). Control PCR of the parental structure (strain R2350) using primer pairs *codY*1-*codY*2 (fragment/lane 1*, 2641 bp) and *amiF*1-*kan*1 (fragment/lane 6*, 1684 bp).

B. PCR probing of the structure of an *Spc*^R transformant (strain R2425) carrying the *codY::spc*^{3A} minitransposon at the CEP-*codY* locus using the following primer pairs: *codY*1-*codY*2 (fragment/lane E, 2641 bp), *amiF*1-*kan*1 (fragment/lane B, 2830 bp), *amiF*1-MP127 (fragment/lane C, 815 bp), and MP127-*kan*1 (fragment/lane D, 917 bp). Control PCR of the parental structure (strain R2350) using primer pairs *codY*1-*codY*2 (fragment/lane F*, 2641 bp) and *amiF*1-*kan*1 (fragment/lane A*, 1684 bp). M, kb ladder.

Fig. 3. Comparing transformation frequencies of *codY*^{spc3/+} in wildtype and *codY*^{+/+} recipient cells.

Chromosomal DNA of strain R2438 (*codY*^{spc3/+} *str41*) was used as donor. Strains used: isogenic *codY*⁺ and *codY*^{+/+} derivatives of R800, R6, and D39, respectively R895 and R2350, R6 and R2427, and D39 and TD73.

Fig. 4. Transformation data indicate the presence of two independent suppressor mutations or a chromosomal rearrangement in the previously described *codY* mutant. Chromosomal DNA of strain TD81 (*codY::spc^{3A} socY str41*) was used as donor. Strains used: isogenic *codY*⁺ and *codY*^{+/+} derivatives of R800-*hex*⁺, R6, D39, and R800-*hex*⁻, respectively R895 and R2350; R6 and R2427; D39 and TD73; R1818 and R2428.

Fig. 5. Effect of *codY* inactivation and of *socY* on spontaneous competence development. Pre-cultures prepared as described in the Experimental procedures were inoculated (1/50th dilution) in C+Y medium (containing 300 µg ml⁻¹ glutamine) and competence (black symbols and curves) and OD (grey symbols and curves) values were recorded throughout incubation at 37°C. Values correspond to individual cultures representative of three independent experiments. Strains used: *codY*⁺ TD82 (panels A-B); *codY socY* mutant TD83 (panels C-D); *socY* mutant TD96 (panels E-F).

Fig. 6. Inactivation of *ami* and *fat* results in tolerance of *codY* inactivation.

A. Distribution of *fatC* and *amiC* mutations identified in strain D39Δ*codY* by whole genome sequence comparison. Positions are given with respect to the first nucleotide of *fatC* (top) and *amiC* (bottom), respectively, taken as +1. The location of the *fatC::spc^{23C} mariner* insertion and *amiC9* mutation used in **panel B** is also indicated.

CYB, CodY binding sites identified in the promoter region of the *fat-fec* and *ami* operons (Hendriksen *et al.*, 2008); -35 and -10 promoter boxes; RRB1-3, RitR binding sites identified

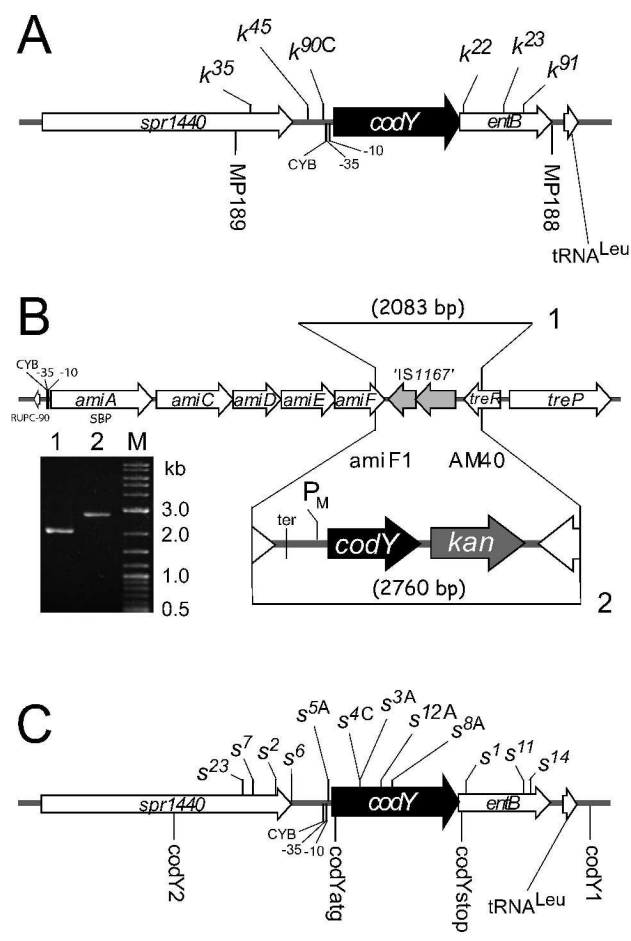
in front of the *fatD* (also called *piuB*) gene (Ulijasz *et al.*, 2004). ABC, ATP-binding cassette protein, MP, membrane protein, SBP, substrate binding protein.

B. The *fatC amiC* combination of mutations allows tolerance of *codY* inactivation.

Chromosomal DNA of strain TD80 (*codY::trim str41*) and TD81 (*codY::spc^{3A} str41*) was used as donor and Str^R as well as, respectively, Trim^R and Spc^R transformants were scored. Strains used: wt, R246; *fatC::cat^{23C}* mutant, R3002; *amiC9* mutant, R3003; *amiC9-fatC::cat^{23C}* double mutant, R3004.

Fig. 7. Iron toxicity may account for inviability of *codY* mutant cells in *S. pneumoniae*.

CodY and RitR share regulatory targets: CodY is a repressor of *ami*, *obl* and *fat-fec*, but activates *dpr*, which encodes an iron-storage peroxide resistance protein (Hendriksen *et al.*, 2008); RitR directly represses *fatD* (or *piuB*) and inactivation of *ritR* derepresses *amiC* while reducing *dpr* expression (Ulijasz *et al.*, 2004). We propose that inactivation of *codY* is not tolerated because it results in simultaneous derepression of iron uptake via Fat-Fec and possibly of heme utilization via Ami (see Discussion), as well as depletion of Dpr, which is required to protect cells from iron toxicity. ILV, isoleucine-leucine-valine (CodY activating branched chain aminoacids).



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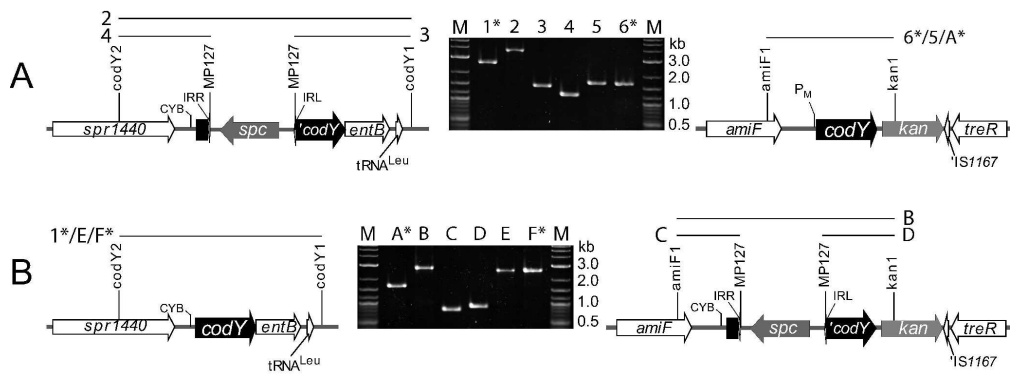


FIG. 2. Caymaris *et al.*

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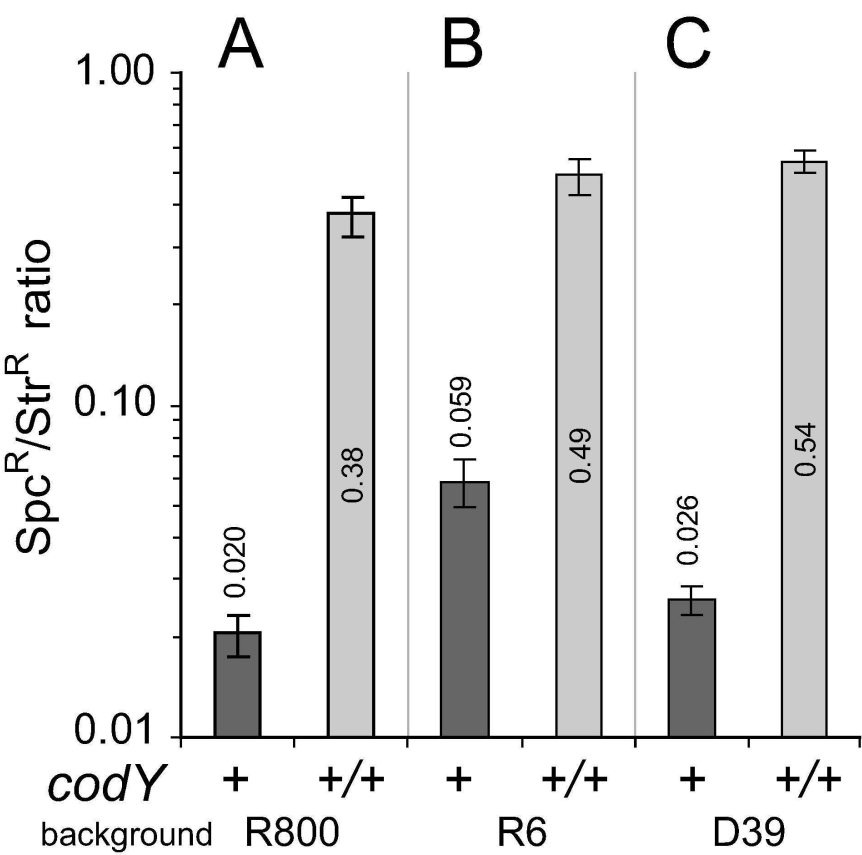


FIG. 3. Caymaris *et al.*

83x111mm (600 x 600 DPI)

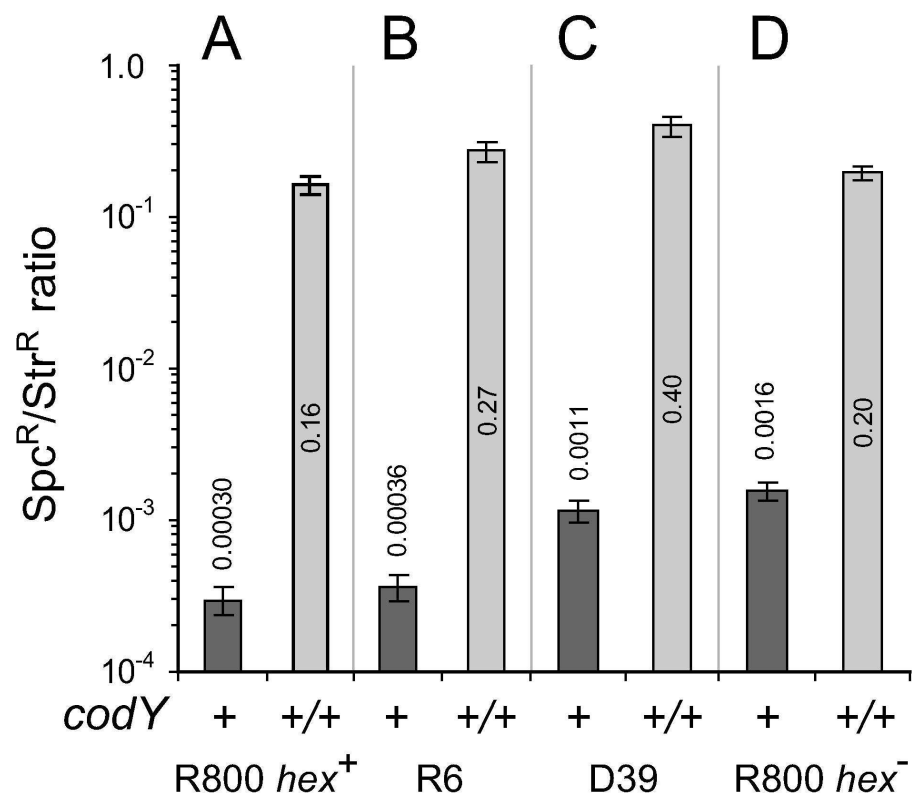


FIG. 4. Caymaris *et al.*

82x106mm (600 x 600 DPI)

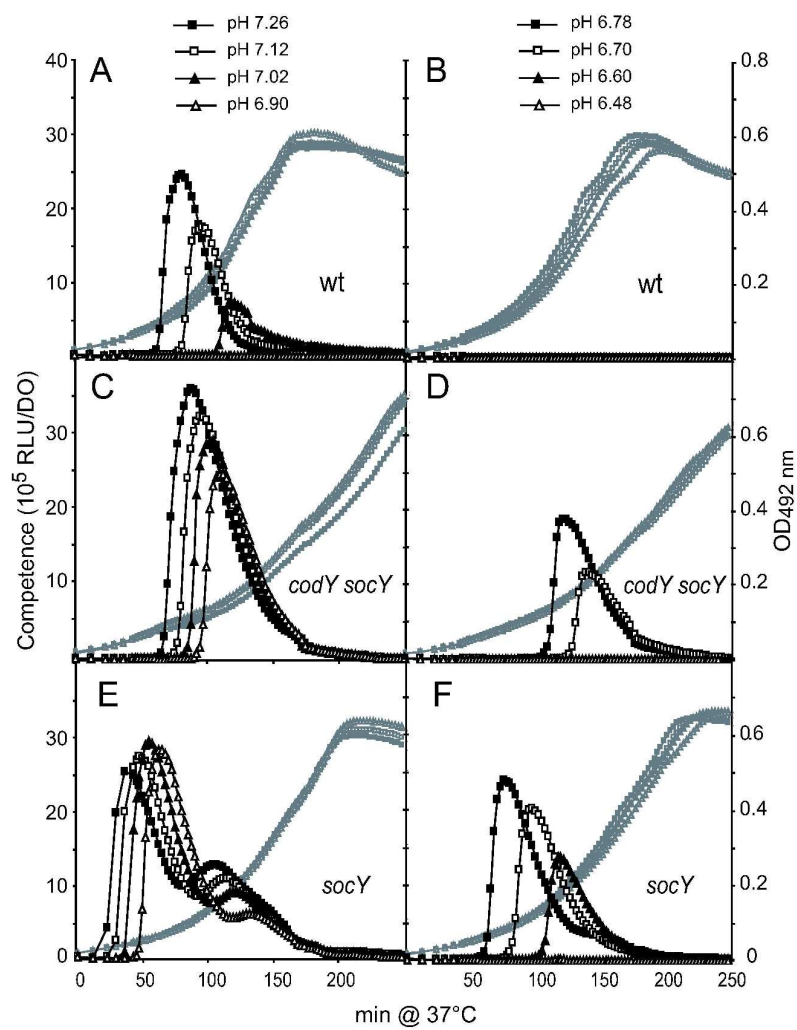


FIG. 5. Caymaris *et al.*

111x168mm (600 x 600 DPI)

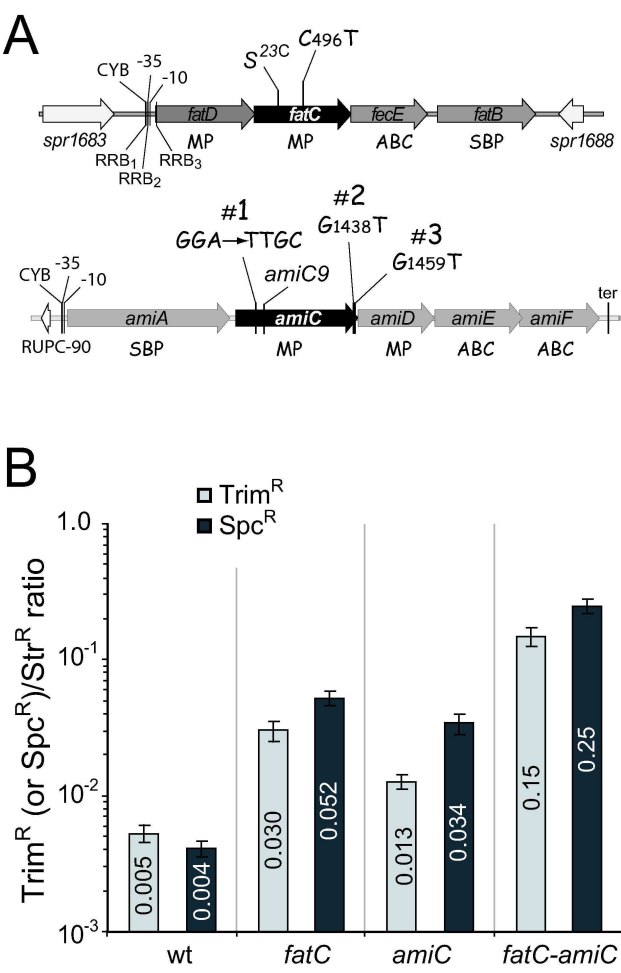


FIG. 6. Caymaris *et al.*

80x154mm (600 x 600 DPI)

