



Evaluation of the Role of the *opgGH* Operon in *Yersinia pseudotuberculosis* and Its Deletion during the Emergence of *Yersinia pestis*

Kévin Quintard, Amélie Dewitte, Angéline Reboul, Edwige Madec, Sébastien Bontemps-Gallo, Jacqueline Dondeyne, Michaël Marceau, Michel Simonet, Jean-Marie Lacroix, Florent Sebbane

► To cite this version:

Kévin Quintard, Amélie Dewitte, Angéline Reboul, Edwige Madec, Sébastien Bontemps-Gallo, et al.. Evaluation of the Role of the *opgGH* Operon in *Yersinia pseudotuberculosis* and Its Deletion during the Emergence of *Yersinia pestis*. *Infection and Immunity*, 2015, 83 (9), pp.3638-3647. 10.1128/IAI.00482-15 . hal-02947674

HAL Id: hal-02947674

<https://hal.science/hal-02947674>

Submitted on 24 Sep 2020

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Evaluation of the Role of the *opgGH* Operon in *Yersinia pseudotuberculosis* and Its Deletion during the Emergence of *Yersinia pestis*

Kévin Quintard,^{a,b,c,d,e,f} Amélie Dewitte,^{a,b,c,d,e} Angéline Reboul,^{a,b,c,d,e} Edwige Madec,^f Sébastien Bontemps-Gallo,^f Jacqueline Dondeyne,^f Michaël Marceau,^{a,b,c,d,e} Michel Simonet,^{a,b,c,d,e} Jean-Marie Lacroix,^f Florent Sebbane^{a,b,c,d,e}

Plague and *Yersinia pestis* Group, INSERM U1019, Lille, France^a; Centre National de la Recherche Scientifique UMR8204, Lille, France^b; Institut Pasteur de Lille, Centre d'Infection et d'Immunité de Lille, Lille, France^c; Université Lille Nord de France, Lille, France^d; Université du Droit et de la Santé de Lille, Centre d'Infection et d'Immunité de Lille, Lille, France^e; Unité de Glycobiologie Structurale et Fonctionnelle, UMR CNRS 8576, Université des Sciences et Technologies de Lille, Université de Lille Nord de France, Villeneuve d'Ascq, France^f

The *opgGH* operon encodes glucosyltransferases that synthesize osmoregulated periplasmic glucans (OPGs) from UDP-glucose, using acyl carrier protein (ACP) as a cofactor. OPGs are required for motility, biofilm formation, and virulence in various bacteria. OpgH also sequesters FtsZ in order to regulate cell size according to nutrient availability. *Yersinia pestis* (the agent of flea-borne plague) lost the *opgGH* operon during its emergence from the enteropathogen *Yersinia pseudotuberculosis*. When expressed in OPG-negative strains of *Escherichia coli* and *Dickeya dadantii*, *opgGH* from *Y. pseudotuberculosis* restored OPGs synthesis, motility, and virulence. However, *Y. pseudotuberculosis* did not produce OPGs (i) under various growth conditions or (ii) when overexpressing its *opgGH* operon, its *galUF* operon (governing UDP-glucose), or the *opgGH* operon or Acp from *E. coli*. A Δ *opgGH* *Y. pseudotuberculosis* strain showed normal motility, biofilm formation, resistance to polymyxin and macrophages, and virulence but was smaller. Consistently, *Y. pestis* was smaller than *Y. pseudotuberculosis* when cultured at $\geq 37^\circ\text{C}$, except when the plague bacillus expressed *opgGH*. *Y. pestis* expressing *opgGH* grew normally in serum and within macrophages and was fully virulent in mice, suggesting that small cell size was not advantageous in the mammalian host. Lastly, *Y. pestis* expressing *opgGH* was able to infect *Xenopsylla cheopis* fleas normally. Our results suggest an evolutionary scenario whereby an ancestral *Yersinia* strain lost a factor required for OPG biosynthesis but kept *opgGH* (to regulate cell size). The *opgGH* operon was presumably then lost because OpgH-dependent cell size control became unnecessary.

Yersinia pestis is the bacterium that causes plague, a fatal disease that cycles between mammalian and flea hosts (1). After *Y. pestis* is taken up into a flea's gut during a blood meal, the bacterium forms a biofilm that ultimately obstructs the digestive tract. The "blocked" (and thus starving) flea will bite a new host many times in an effort to feed. During these unproductive attempts to feed, some bacteria are dislodged from the biofilm and regurgitated into the dermal biting site (2–4). Although it is not clear whether these *Y. pestis* organisms are truly phagocytized, it generally assumed that they replicate initially within phagocytes and produce antiphagocytic factors; this leads to extracellular replication throughout the draining lymph node and ultimately in the blood and other deep tissues (5).

Y. pestis emerged from *Yersinia pseudotuberculosis*, a widely spread environmental bacterium that causes a mild bowel disease in humans following ingestion of contaminated foods (6). During its emergence, *Y. pestis* accreted a small amount of genetic material via horizontal transfer but also lost a large number of functional genes (7). Early investigations showed that the emergence of plague can be explained (at least in part) by the acquisition of genetic material (8–11). Hence, a stepwise scenario in which sequential gene losses led to a flea-borne transmission route was subsequently proposed (12, 13). However, the complete set of genetic events that led to the ability to cause plague remains unknown.

Genomic analysis has indicated that in comparison with all strains of *Y. pseudotuberculosis* for which the genome has been sequenced, all *Y. pestis* strains lack a 12-kb block encompassing 8

genes (including the *opgGH* locus) (see Fig. S1 in the supplemental material). The latter operon is found in many gammaproteobacteria and is functionally homologous to the *nvbAB*, *chvAB*, and *cgs* loci found in alphaproteobacteria (14–27). The *opgGH* operon encodes glucosyltransferases that synthesize branched glucans from UDP glucose (UDP-Glc), using an acyl carrier protein (ACP) as a cofactor (28, 29). In most bacterial species, the rate of glucan synthesis decreases in proportion to the osmolarity of the external microenvironment (20, 21, 25, 26, 28, 30–33). Accordingly, these macromolecules are generally referred to as osmoregulated periplasmic glucans (OPGs). It is noteworthy that *Bru-*

Received 13 April 2015 Returned for modification 20 May 2015

Accepted 26 June 2015

Accepted manuscript posted online 6 July 2015

Citation Quintard K, Dewitte A, Reboul A, Madec E, Bontemps-Gallo S, Dondeyne J, Marceau M, Simonet M, Lacroix J-M, Sebbane F. 2015. Evaluation of the role of the *opgGH* operon in *Yersinia pseudotuberculosis* and its deletion during the emergence of *Yersinia pestis*. *Infect Immun* 83:3638–3647. doi:10.1128/IAI.00482-15.

Editor: B. A. McCormick

Address correspondence to Jean-Marie Lacroix, jean-marie.lacroix@univ-lille1.fr, or Florent Sebbane, florent.sebbane@inserm.fr.

J.-M.L. and F.S. are co-senior authors.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.00482-15>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.00482-15

cella and *Rhizobiaceae* may secrete OPGs into the external environment (15, 34).

Hence, in some (but not all) bacteria, OPGs may act as osmolytes that maintain turgor pressure during growth under low-osmolarity conditions (27). In gammaproteobacteria, OPGs reportedly control not only cell motility, biofilm formation, and virulence (via the regulation of the Rcs regulatory system) but also protein folding and degradation and carbohydrate catabolism (via as-yet-unknown mechanisms) (16, 24, 35–40). Furthermore, OPGs can sequester antibiotics and detergents, thus protecting the bacterium against these harmful molecules (40–42). When secreted in the environment, OPGs were found to prevent phagolysosome biogenesis and thus enable intracellular replication of *Brucella* (34, 43). OPGs are also involved in nodule production in plants that host symbiotic bacteria (22, 23, 32, 44, 45). Hence, in all bacterial species studied to date, OPGs appear to be key players in virulence and in bacterial adaptation to environmental change.

Lastly, OpgH controls bacterial cell size and shape in an UDP-Glc concentration-dependent manner (independently of its glucosidase activity and thus its effect on the Rcs system) (46). In response to an increase in the intracellular concentration of UDP-Glc (i.e., passage into a nutrient-rich environment), OpgH reduces the rate of the cytokinetic ring formation by directly sequestering the cell division protein FtsZ at midcell. This results in a greater cell size but does not affect the mass doubling time. Hence, OpgH's sequestering activity might provide a selective advantage in response to changes in nutrient availability, because bacterial cell shape is considered to have selective value (47). However, this idea remains to be proven.

Given this context, the absence of *opgGH* in *Y. pestis* is intriguing because the bacterium is much more virulent than *Y. pseudotuberculosis* and forms biofilms for efficient flea-borne transmission (8). Hence, we sought to establish why *Y. pestis* has lost the *opgGH* operon and whether this loss contributed to the emergence of modern plague. However, we first had to study *opgGH*'s previously unknown role in *Y. pseudotuberculosis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in the present study are listed in Table S1 in the supplemental material. Lysogeny broth (LB) with or without sodium chloride (NaCl), low-osmolarity medium (LOS) (21), and LB agar with or without kanamycin (50 µg/ml) or carbenicillin (100 µg/ml) supplementation were used to culture bacteria at the desired temperature. *opgGH*-negative *Y. pseudotuberculosis* mutants (in which bases 1070 to 1829 are deleted) were generated using the pCVD442-based method and checked in a PCR analysis, as previously described (48). The *opgGH* and *galUF* operon from *Y. pseudotuberculosis* and the *acpP* gene from *Escherichia coli* (under the control of their respective promoters) were amplified by PCR using the primer sets Opg1 (5'-CGCGCTGCAGCTGACTTGCTAGGCTTATGC-3') plus Opg2 (5'-GTGTGGATCCCTCTTATGGTCTGCCGCTAC-3'); Gal1 (5'-AAAAGAGCTCAGATGTTGGATATTATTTTCA-3') plus Gal2 (5'-ACATGCATGCACCGCCATACCGACAACGCCGA-3'); and Acp1 (5'-CCGCGGATCCTGATTGCGTTATTGGGGGG-3') plus Acp2 (5'-CCCGGAATTCTAAACTCAGGCGGTGCAAC-3'). The *opgGH* and *galUF* amplicons were digested with the restriction enzymes BamHI/PstI and EcoRI/SphI, respectively, and were thus inserted into the corresponding restriction sites of the pUC18Not and pBR322 vectors (yielding the plasmids pNF400 and pRB100). The *acpP* amplicon was cloned into the pCRII cloning vector (Invitrogen) to yield pAcpEc. Lastly, pNF400 was used to subclone the *Y. pseudotuberculosis opgGH* operon at the BamHI

and PstI sites in the mini-Tn7 pUC842 vector and thus integrate the operon into the *Y. pestis* chromosomal at *att* Tn7 (49).

Extraction of OPGs. OPGs were extracted as previously described (28). Briefly, bacteria cultured under the chosen growth conditions were pelleted by centrifugation, suspended in sterile, distilled water, and then lysed with trichloroacetic acid. After centrifugation of the lysate, the supernatant was collected and mixed with charcoal. After incubation at room temperature with vigorous shaking, the charcoal (which bound the OPGs) was collected by centrifugation and mixed with pyridine to release the glucans. After centrifugation, the OPG-containing supernatant was concentrated by rotary evaporation and then fractionated by gel filtration. The presence of OPGs in the eluate fractions was monitored using the anthrone-sulfuric acid method (50). The putative secretion of OPGs into the growth media was determined by applying the above-described procedure to the culture supernatant.

Motility assay. Bacteria grown overnight in LB at 21°C (for *Y. pseudotuberculosis*) or 30°C (for *E. coli* and *Dickeya dadantii* [previously *Erwinia chrysanthemi*]) were diluted in LB to an optical density at 540 nm (OD₅₄₀) of 0.2. Five microliters of the freshly diluted suspension was spotted at the center of an LB soft agar plate containing 0.25% agar (for *Y. pseudotuberculosis*) or 0.4% agar (for *E. coli* and *D. dadantii*). Bacterial swimming diameters were measured after a 24-hour incubation at 21°C (for *Y. pseudotuberculosis*) or at 30°C (for *E. coli* and *D. dadantii*).

Biofilm assay. Biofilms were assayed as previously described, with slight modifications (12). Briefly, bacteria suspended in LB supplemented with 4 mM MgCl₂ and 4 mM CaCl₂ was added to the wells of a 24-well plate. After a 24-hour incubation at 21°C with shaking, the planktonic bacteria were rinsed away with water and the attached bacterial biofilm was stained with a crystal violet dye solution. After staining, the wells were washed with water. The dye attached to the biofilm was then released by the addition of an ethanol-acetone solution. The absorbance of each well was measured at 540 nm. Measurements were corrected by subtracting the crystal violet binding observed for control (noninoculated) wells. The absorbance ratio for each strain of interest (relative to the parental strain) was calculated.

Analysis of *opgGH* expression. In transcription assays, cultures were mixed with RNeasy Protect reagent (Qiagen) according to the manufacturer's protocol and then pelleted for RNA extraction with the Nucleospin RNA kit (Macherey-Nagel). RNA samples were (i) reverse transcribed using the Superscript III kit (Invitrogen) after having been treated twice with DNase (using a DNA-free kit from Ambion), (ii) checked for integrity (using an Agilent Bioanalyzer), and (iii) checked for the absence of DNA contamination (using PCR). Quantitative PCR was performed using SYBR green technology with the primer sets *opg3* (5'-GCTGACGGT AATAGCCATTCCAT-3') plus *opg4* (5'-CCCGTTTGCATCAGCAGAT T-3') and *crr1* (5'-GCCCTCTGGCAATAAA ATGG-3') plus *crr2* (5'-A GCATGGTTGGTCTCGAAAATT-3'). Data were normalized against levels of the *crr* transcript, and the relative fold change was calculated using the $\Delta\Delta C_T$ method.

In translation assays, whole-cell lysates were separated by sodium dodecyl sulfate-polyacrylamide (10%) gel electrophoresis and then electrophoretically transferred to a nitrocellulose membrane using equipment and protocols from Bio-Rad Laboratories. The membrane was blocked overnight at 4°C with 5% (wt/vol) nonfat dry milk in phosphate-buffered saline containing 0.2% Tween 20 (PBS-T), washed three times for 10 min with PBS-T, probed at room temperature for 1.5 h with a rabbit polyclonal against His-tagged OpgG from *E. coli* (Eurogentec) diluted in PBS-T, and then washed three times with PBS. Immunoreactive proteins were detected using a horseradish peroxidase-conjugated goat anti-rabbit IgG and the ECL plus Western blotting kit (GE Healthcare), according to the manufacturer's protocols.

Measurement of bacterial cell size. Smears of mid-log-phase bacterial cultures in LB supplemented with glucose (2 g/liter) were air dried and ethanol fixed on glass slides. Fixed bacteria were successively washed with

70% ethanol, stained with fuchsin, and then washed with water. The cell length (250 cells per slide) was measured using ImageJ software.

Interactions with serum, polymyxin, and macrophages. Survival and growth upon contact with serum, the MIC of polymyxin, and survival within macrophages were assayed as previously described (51). Briefly, a bacterial suspension of 5×10^8 bacteria/ml in PBS was prepared from an overnight culture in LB at 21°C. The suspension was diluted 10-fold into fresh human serum and then incubated at 37°C. Bacterial growth was measured by counting the CFU. To determine the MIC of polymyxin, a range of polymyxin concentrations were added to inocula of 10^6 bacteria/ml (prepared from an overnight culture at 21°C or 37°C in LB). The MIC was measured after a 24-hour incubation. For the assay of survival within phagocytes, bacteria from an overnight culture at 21°C were suspended in Dulbecco's modified Eagle's medium (DMEM) medium and added to RAW macrophages suspended in DMEM at multiplicity of infection of 10. Cells were centrifuged for 5 min at $18 \times g$ and then incubated at 37°C in a 5% CO₂ atmosphere. Thirty minutes after contact, nonphagocytized bacteria were rinsed away with DMEM, and fresh DMEM supplemented with gentamicin was then added. At different time points, the supernatant was removed, cells were lysed with cold water on ice, and serial dilutions of the lysate were spread on blood agar to count CFU.

Determination of bacterial virulence. Groups of six to eight 9-week-old OF-1 female mice (Charles River) were inoculated (i) via intragastric and intravenous routes with *Y. pseudotuberculosis* strain 2777 or its isogenic *opgGH* mutant or (ii) via the intradermal route with *Y. pestis* CO92 strain or its derivative, as previously described (5, 52). Mice were monitored daily after inoculation. To measure the time course of organ colonization, organs (Peyer's patches, mesenteric lymph nodes, spleens, and livers) were collected in PBS immediately after euthanasia and put on ice. Dilutions of the triturated organs were plated on LB agar for CFU counting after 48 h of incubation at 28°C. The virulence of the phytopathogen *D. dadantii* on chicory leaves was determined as previously described (24). Briefly, a bacterial suspension in saline solution was prepared from an overnight culture in LB medium and placed on a wounded leaf. Maceration was determined after 48 h of incubation in a dew chamber at 30°C. At the time when the present experiments were initiated, they were not subject to ethical approval. However, the legislation changed during the study. Accordingly, some (but not all) of the experiments were approved by the regional animal care and use committee (Lille, France). However, all animal experiments were performed in compliance with French and European regulations on the care and protection of laboratory animals (EC Directive 86/609 and the French Act 2001-486, issued on 6 June 2001).

Flea infection. *Y. pestis* strain KIM6+ and its derivative were cultured in brain heart infusion (BHI) for 18 h at 37°C (without shaking), pelleted by centrifugation, and then suspended to 3×10^8 bacteria/ml in PBS. Next, 1 ml of the suspension was homogenized with 5 ml of heparinized mouse blood. The contaminated blood was added to an artificial feeding system in which ~400 *Xenopsylla cheopis* fleas were allowed to feed for 1 h. After feeding, two cohorts of infected fleas were collected, maintained at 21°C and 75% humidity, and allowed to feed twice a week for a 4-week period. Notably, one cohort (composed of 50 males and 50 females) was used to monitor blockage of the flea gut. The second cohort (composed of females only) was used to measure infection rates and bacterial loads immediately after and 27 days after the infectious blood meal. In particular, 20 insects were individually triturated and plated on BHI containing 1 µg/ml Irgasan and 10 µg/ml hemin. CFU were counted after a 48-hour incubation at 28°C.

Statistical analysis. A one-way analysis of variance, the Gehan-Breslow-Wilcoxon test, and the Mann-Whitney U test were used to analyze the results of *in vitro* experiments, survival curves, and data from flea infection experiments.

RESULTS

In contrast to other proteobacteria, *Y. pseudotuberculosis* does not produce detectable OPGs. The role of *opgGH* in *Y. pseudotuberculosis* was not known prior to the present study. Hence, we first had to determine its role in order to establish whether loss of *opgGH* was likely to have been selected during the emergence of plague or, in contrast, had no impact. Surprisingly, no OPGs could be detected in *Y. pseudotuberculosis* strain 2777 grown under low-osmolarity conditions (NaCl-free LB and LOS) when the assay method conventionally employed for all bacterial species studied to date (including *E. coli* and *D. dadantii*, which were used as positive controls here) was used (Fig. 1A and data not shown). No OPGs were detected in cultures of *Y. pseudotuberculosis* strain 2777 or in the extracellular medium when the extraction volume was increased 10-fold (i.e., up to 1 liter of culture medium) (data not shown). These results were not due to an artifact with strain 2777, because we did not detect any OPGs for the unrelated *Y. pseudotuberculosis* strain 2515. However, Western blot experiments indicated that *Y. pseudotuberculosis* does translate the *opgGH* operon (Fig. 2B).

The *opgGH* operon from *Y. pseudotuberculosis* encodes functional OPGs in *E. coli* and *D. dadantii*. The above-mentioned, unexpected results prompted us to determine whether the *opgGH* operon from *Y. pseudotuberculosis* encodes functional enzymes. To this end, we looked at whether *opgGH* from *Y. pseudotuberculosis* restores the phenotypes associated with the loss of OPG synthesis in *E. coli* and *D. dadantii*. In the absence of OPGs, both *E. coli* and *D. dadantii* show motility defects, and *D. dadantii* is also avirulent on chicory leaves (24, 38). OPGs were purified from OPG-deficient strains (grown in NaCl-free LB) of *E. coli* (*opgG*-negative, *opgH*-negative, and *opgGH*-negative strains) and *D. dadantii* (an *opgG*-negative strain), in which the *opgGH* operon from *Y. pseudotuberculosis* was expressed under the control of its own promoter (Fig. 1A and data not shown). We found that motility was restored in both *E. coli* and *D. dadantii* *opgG*-mutant strains and that *D. dadantii* was newly virulent on chicory leaves (Fig. 1B and C).

***Y. pseudotuberculosis* constitutively transcribes *opgGH* but does not produce detectable OPGs under various growth conditions.** Given that *opgGH* from *Y. pseudotuberculosis* does indeed encode functional enzymes, we next sought to identify growth conditions that could stimulate OPG production, i.e., whether synthesis of OPGs is regulated at transcriptional and/or posttranscriptional level in *Y. pseudotuberculosis*. To this end, we monitored (i) the transcription of *opgGH* using quantitative reverse transcription-PCR (qRT-PCR) and (ii) levels of the OpgGH glucosyltransferase product under various growth conditions (high-, low-, and medium-osmolarity media; 21°C and 37°C; aerobic and anaerobic conditions; and heat-inactivated serum [which was used here as a surrogate model of *in vivo* conditions]). The assay results indicated that *opgGH* was constitutively transcribed under all the conditions tested (Fig. 2A) but that no OPGs were detected.

Overexpression of *opgGH* by *Y. pseudotuberculosis* does not induce OPG biosynthesis. The above data suggested that *Y. pseudotuberculosis* lacks essential OPG synthesis factors and/or carries genes encoding inhibitors of OPG biosynthesis. To distinguish between these two possibilities, we decided to screen for OPGs in *Y. pseudotuberculosis* overexpressing *opgGH* (since genetic overex-

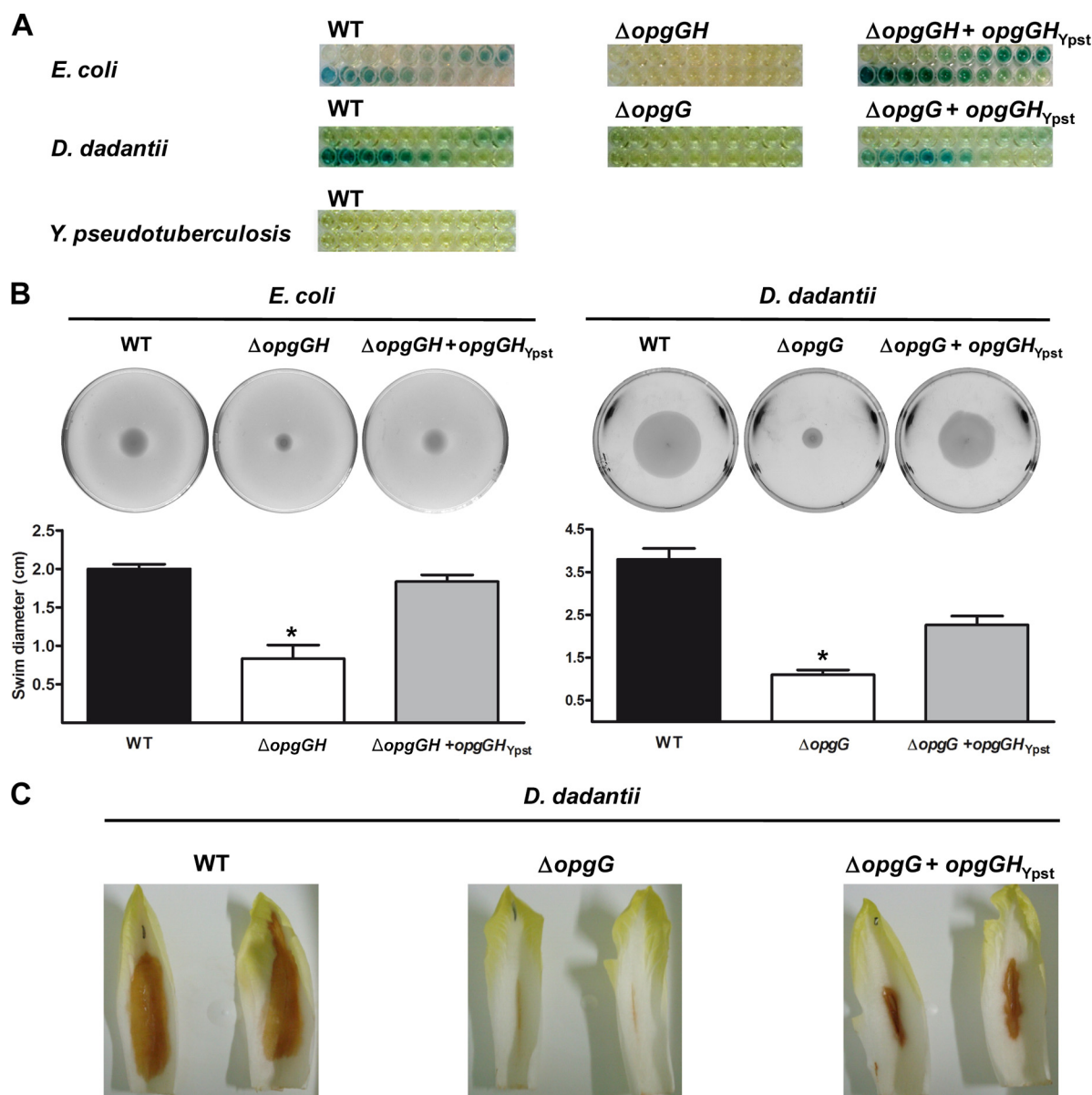


FIG 1 *Y. pseudotuberculosis* does not produce detectable OPGs, despite the fact that its *opgGH* operon encodes functional active glucosyltransferases in *E. coli* and *D. dadantii*. (A) Eluate fractions of bacterial samples (prepared for OPG extraction, as described in Materials and Methods) stained with anthrone-sulfuric acid reagent. Blue color indicates the presence of OPGs. In these experiments, WT *Y. pseudotuberculosis*, *E. coli*, and *D. dadantii* strains and their derivative *opgGH*- or *opgG*-negative strains harboring (or not) the *Y. pseudotuberculosis* *opgGH* operon and its own promoter on a plasmid (pNF400; +*opgGH*_{Ypst}) were grown in NaCl-free LB. (B) Swimming motility of WT *E. coli* and *D. dadantii* strains (black bars) and their OPG-negative derivative strains harboring pNF400 (+*opgGH*_{Ypst}) (gray bars) or not (white bars), after a 24-hour incubation on a LB soft agar plate at 30°C. The photos are representative of bacteria after 24 h of swimming, and the swim diameter (mean $\pm$ SEM) was determined from three independent experiments. The swim diameters of the ΔopgGH *E. coli* strain and the ΔopgG *D. dadantii* strain were significantly lower than those of the corresponding WT strain and the respective OPG-deficient mutant strain expressing *opgGH* from *Y. pseudotuberculosis* ($P < 0.05$ in a one-way analysis of variance). However, partial complementation was observed in *D. dadantii*, presumably due to plasmid instability in this species. (C) Virulence of *D. dadantii* on chicory leaves after 48 h of contact. Leaves were infected with 10^7 CFU of WT *D. dadantii*, ΔopgG mutant, or ΔopgG mutant harboring pNF400 (+*opgGH*_{Ypst}). *, $P < 0.05$.

pression can override inhibition mechanisms). We therefore transformed *Y. pseudotuberculosis* with a high-copy-number plasmid (pUC18) containing *opgGH* (under the control of its own promoter), confirmed (using qRT-PCR) that the operon is highly transcribed, and then assayed the transformed strain for OPG synthesis. Even when the recombinant strain overexpressed *opgGH* 100-fold more intensely than the parental strain, OPGs were still not detected (Fig. 2A and data not shown). Lastly, OPG synthesis

was not detected when *Y. pseudotuberculosis* overexpressed *opgGH* from *E. coli* (under the control of its own promoter); the data were identical to those shown in Fig. 1A. This finding suggests that *Y. pseudotuberculosis* lacks factors that are important for OPG biosynthesis.

The absence of detectable OPGs in *Y. pseudotuberculosis* is not due to a lack of functional ACP. The ACP is essential for OPG synthesis in *E. coli* (29), and certain amino acid substitutions can

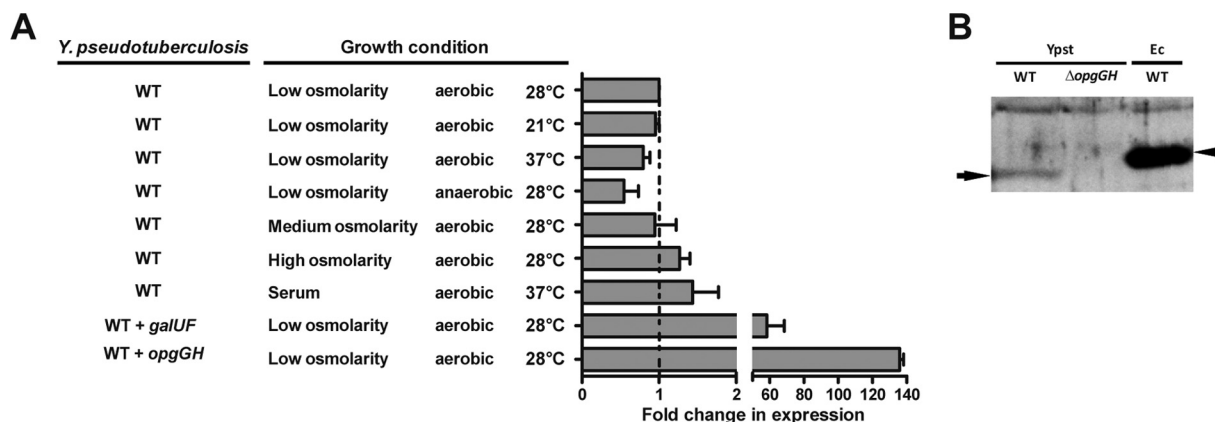


FIG 2 *Y. pseudotuberculosis* constitutively expresses the *opgGH* operon under various growth conditions. (A) Fold change expression of *opgGH* in *Y. pseudotuberculosis* (strain 2777) grown under various conditions and in *Y. pseudotuberculosis* harboring a recombinant pBR322 plasmid containing its *galUF* operon or a recombinant pUC18 plasmid containing its *opgGH* operon, compared with the *opgGH* expression level in *Y. pseudotuberculosis* grown under low-osmolarity and aerobic conditions and at 28°C. *galUF* and *opgGH* were under the control of their own respective promoters. Under each condition, the level of *opgGH* transcripts was normalized to that of *crr* transcripts. The data are means and SEM from two independent experiments. (B) Immunoblot of a whole-cell lysate of *Y. pseudotuberculosis* (strain 2777), its isogenic Δ *opgGH* mutant and WT *E. coli* grown in LB at 30°C. *E. coli* was used as a positive control. The blot was immunostained with a polyclonal antibody against *E. coli* OpgG. Arrowheads indicate the positions of the *Y. pseudotuberculosis* OpgG protein.

affect the protein's ability to participate in OPG biosynthesis (14). Hence, our inability to detect any OPGs in *Y. pseudotuberculosis* might have been caused by a nonfunctional ACP. Consistently, an amino acid sequence alignment revealed that ACP from *Y. pseudotuberculosis* and *E. coli* diverge (see Fig. S2 in the supplemental material). However, we did not detect any OPGs in *Y. pseudotuberculosis* expressing *acpP* (under the control of its own promoter) from *E. coli* (data not shown). Hence, ACP does not appear to account for the lack of OPGs.

The absence of detectable OPGs in *Y. pseudotuberculosis* is not due to a defect in UDP-Glc synthesis. UDP-glucose is an essential precursor for the biosynthesis of several polysaccharides, including OPGs (27). Hence, one can hypothesize that *Y. pseudotuberculosis* does not produce enough UDP-glucose to drive the production of OPG. To test this hypothesis, the *galUF* operon (governing UDP-Glc synthesis [53]) was overexpressed (using the average-copy-number plasmid pBR322) in *Y. pseudotuberculosis*. This overexpression strongly induced the transcription of the *opgGH* operon but did not lead to the production of detectable OPGs (Fig. 2A and data not shown), further supporting the hypothetical lack of essential factors for OPG biosynthesis.

The loss of *opgGH* does not impact Rcs-dependent phenotypes in *Y. pseudotuberculosis*. The above data suggested that *Y. pseudotuberculosis* does not synthesize OPGs. However, we could not rule out the presence of very small amounts of extremely active OPGs (or other glucans) that might nevertheless control the Rcs regulatory system. Hence, we next looked at whether a Δ *opgGH* *Y. pseudotuberculosis* strain displayed one or more phenotypes (defects in motility, biofilm formation, and virulence) attributed to aberrant stimulation of the Rcs system by OPGs (24, 36, 38). As shown in Fig. 3A and B, Δ *opgGH* *Y. pseudotuberculosis* remained fully motile and formed the same amount of biofilm as the wild-type (WT) strain. Furthermore, the mutant grew normally within macrophages (Fig. 3C). The MICs of polymyxin for the mutant grown at 21 and 37°C were identical to those observed for the parental strain (48 μ g/ml and 192 μ g/ml, respectively), suggesting that the absence of *opgGH* did not impact virulence.

Consistently, the time course of organ colonization in animals inoculated by intragastric and intravenous routes revealed that the mutant colonized the Peyer's patches, the mesenteric lymph nodes, the spleen, and the liver at much the same rate as the WT strain (Fig. 3D and E).

Loss of *opgGH* during emergence probably explains why *Y. pestis* is smaller than *Y. pseudotuberculosis*. Independently of its ability to synthesize OPGs, OpgH is involved in elaboration of the bacterial cell's architecture by sequestering FtsZ and thus affecting the cell length (46). Hence, we looked at whether the loss of *opgGH* in *Y. pseudotuberculosis* affects bacterial shape during growth in LB supplemented with glucose and at the temperatures encountered by the bacterium in nature (21°C and 37°C). Regardless of the growth temperature, the *opgGH* mutant was significantly (11% to 14%) smaller than the WT strain (Fig. 4). However, the cell size was normal when the mutant was complemented with the WT *opgGH* operon. Consistently, *Y. pestis* (which lost *opgGH* during emergence) was significantly (~11%) smaller than *Y. pseudotuberculosis* when cultured at 37°C. Furthermore, expression of the *Y. pseudotuberculosis* *opgGH* operon from the chromosomally integrated mini-Tn7 increased the length of *Y. pestis* to the same size as its recent ancestor (1.64 μ m and 1.66 μ m, respectively). Surprisingly, *Y. pestis* grown at 21°C was the same size as *Y. pseudotuberculosis* and was greater still (by 8%) when the bacillus expressed *opgGH* from *Y. pseudotuberculosis*. However, in a one-way analysis of variance, the latter difference was not statistically significant. Taken as a whole, and considering that OpgH (but not OpgG) was found to control cell size in *E. coli* (46), these data suggest that (i) *opgH* controls the length of *Y. pseudotuberculosis* and (ii) deletion of *opgH* during the emergence of *Y. pestis* decreased the bacterial cell size at the mammalian host temperature but not at a temperature required for efficient flea-borne transmission.

Effect of the expression of *opgGH* from *Y. pseudotuberculosis* on the *Y. pestis* life cycle. A particular bacterial shape is thought to have selective value by facilitating specific cell functions (47). Hence, we wondered whether the loss of *opgGH* provided *Y. pestis* with a selective advantage for the emergence of flea-borne plague.

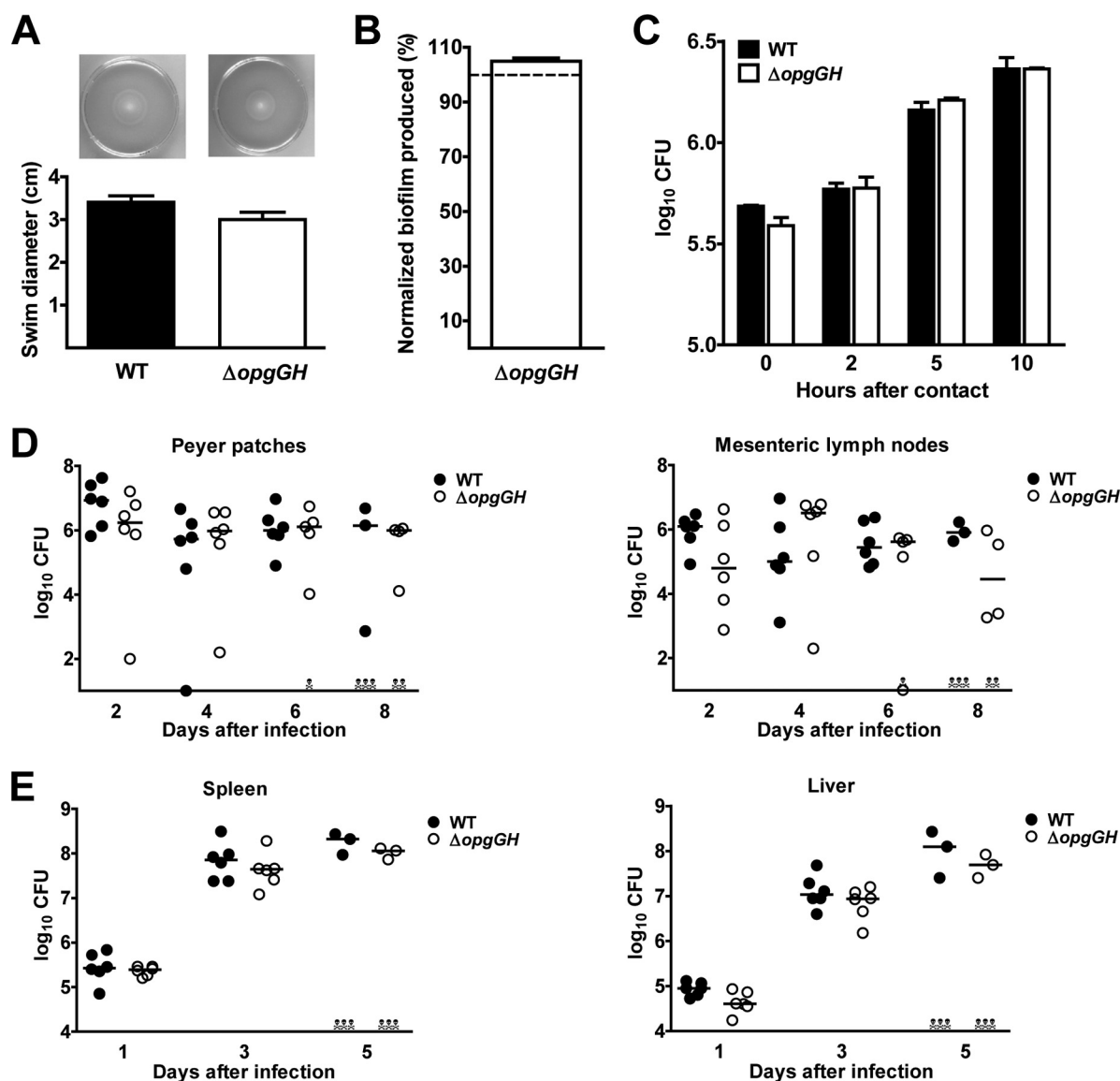


FIG 3 In contrast to other proteobacteria, *Y. pseudotuberculosis* lacking a functional *opgGH* operon does not show the pleiotropic phenotype resulting from the activity of the OPGs through the Rcs signaling pathway. Data on swimming motility on an LB soft agar plate after a 24-hour incubation at 21°C (A), biofilm formation (relative to the WT strain) after a 24-hour incubation at 21°C with shaking in LB supplemented with Ca^{2+} and Mg^{2+} (B), rates of survival within RAW 264.7 macrophages up to 10 h after internalization (C), the time course of colonization of the Peyer's patches and mesenteric lymph nodes after intragastric inoculation (10^3 bacteria) (D), and the time course of colonization of the spleen and liver after intravenous inoculation (10^3 bacteria) by the WT strain 2777 and its derivative $\Delta opgGH$ strain (E) are shown. The photos are representative of bacteria after 24 h of swimming. The means and SEM from three independent experiments (A and B) and two independent experiments (C) are shown. The time course of organ colonization was determined from one experiment using groups of six animals (D and E); horizontal lines indicate the medians of the individual data points. Bacterial loads in organs did not vary significantly according to the presence or absence of *opgGH* ($P > 0.05$ in a one-way analysis of variance).

Virulence of *Y. pestis* containing a copy of *opgGH* at the *att* Tn7 chromosomal site was assessed in a mouse model of bubonic plague and in the *Xenopsylla cheopis* rat flea model. Regardless of the presence or absence of *opgGH* in the *Y. pestis* genome, all mice inoculated with ~ 10 CFU succumbed to the disease. A slight increase in the median survival time was observed for animals inoculated with *Y. pestis* expressing *opgGH* (4 days, versus 3 in *Y. pestis* not expressing *opgGH*), although the survival curves were not significantly different ($P > 0.3$). Consistently, we found that expression of *opgGH* did not affect the bacterium's capability to survive within macrophages or to grow in normal human serum (see Fig.

S3 in the supplemental material). Furthermore, expression of *opgGH* did not affect the bacillus' capability to block the flea digestive tract (Fig. 5A). Accordingly, 27 days after the infected blood meal, the percentage of infected fleas and the bacterial loads recovered from the infected fleas did not differ according to the expression or nonexpression of *opgGH* (Fig. 5B and C). These data are line with the fact that biofilm formation *in vitro* was not affected in *Y. pestis* expressing *opgGH* (biofilm formation, reported as the mean $\pm$ standard error of the mean [SEM], was $89 \pm 9.6\%$ relative to the parental strain, as determined using data from two independent experiments).

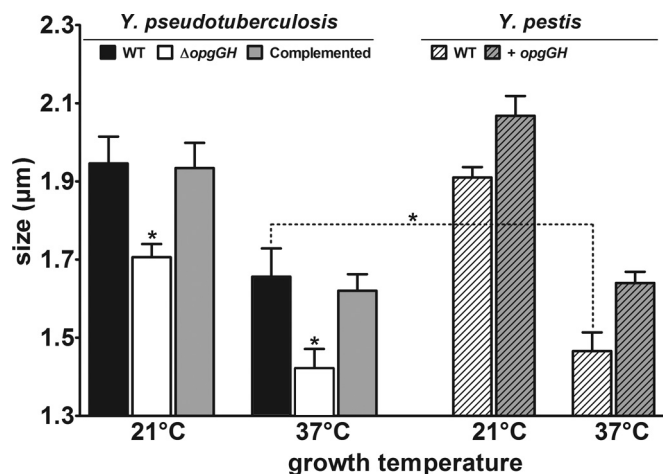


FIG 4 *Y. pestis* is smaller than *Y. pseudotuberculosis* when grown at 37°C (but not at 21°C), presumably because the bacterium lost *opgGH* during its emergence. The lengths of *Y. pseudotuberculosis* WT strain 2777, the Δ *opgGH* strain, the complemented mutant, and *Y. pestis* KIM6+ expressing *opgGH* from *Y. pseudotuberculosis* (+*opgGH*) or not (WT) were measured during exponential-phase growth in LB supplemented with glucose at 21°C and at 37°C. The data are means and SEM from five independent experiments. Regardless of the growth temperature, the Δ *opgGH* *Y. pseudotuberculosis* strain (but not the complemented mutant strain) was significantly smaller than the WT strain ($P < 0.05$ in one way analysis of variance). When grown at 37°C, *Y. pestis* was significantly smaller than *Y. pseudotuberculosis* except when it expressed *opgGH* from *Y. pseudotuberculosis* ($P < 0.05$ in one way analysis of variance). *, $P < 0.05$.

DISCUSSION

All of the 20 or so bacterial species studied to date that carry the *opgGH* operon or one of its functional homologs (*ndvAB*, *chvAB*, and *cgs*) have been found to govern the biosynthesis of the glucose homopolymer OPG. The latter accounts for 0.5% to 10% of the bacterial dry weight (22–24, 32, 34, 36, 40, 44, 45). However, using the conventional method used to extract OPGs from all the bacterial species studied to date, we did not detect any OPGs (i) in *Y. pseudotuberculosis* or its culture supernatant after bacterial growth under various growth conditions, (ii) when the bacterium artificially overexpressed its own *opgGH* operon or that of *E. coli*, (iii) when *Y. pseudotuberculosis* overproduced the Opg enzymes' substrate (UDP-Glc) via the overexpression of *galUF*, or (iv) when the bacterium expressed the *acpP* gene from *E. coli*, which encodes a cofactor required for OPG biosynthesis. Lastly, several cellular processes (motility, biofilm formation, and virulence) known to be controlled by OPGs via the Rcs signaling pathway in *E. coli* and/or *D. dadantii* (24, 38) were unaffected in a Δ *opgGH* *Y. pseudotuberculosis* strain. However, the Rcs system is known to control biofilm formation and motility in *Y. pseudotuberculosis* (54). Taken as a whole, these data suggest that *Y. pseudotuberculosis* has lost the capability to produce OPGs.

One can legitimately wonder why *Y. pseudotuberculosis* (probably) does not produce OPGs, considering that (i) our present data and data from the KEGG database suggest that the bacterium produces the Opg enzymes, the ACP cofactor, and the UDP-Glc substrate (following glucose incorporation or glucogenesis) and (ii) the *opgGH* operon from *Y. pseudotuberculosis* encodes func-

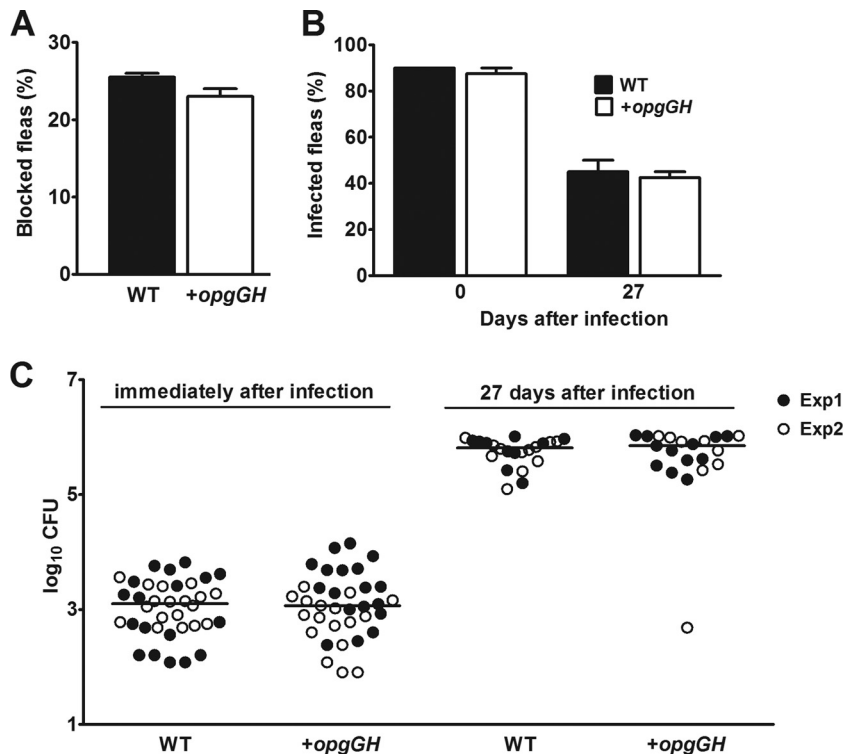


FIG 5 *Y. pestis* expressing *opgGH* from *Y. pseudotuberculosis* is fully competent in fleas. (A) Proportion of fleas became blocked in the 4 weeks following an infected blood meal; (B) proportion of infected fleas; (C) bacterial loads immediately after and 27 days after the infectious meal. Experiments were performed with *Y. pestis* WT KIM6+ (WT) and *Y. pestis* KIM6+ harboring a chromosomally integrated mini-Tn7 encompassing the *opgGH* operon from *Y. pseudotuberculosis* (+*opgGH*). Data in panels A and B are means and standard deviations (SD) from two independent experiments. In panel C, the data correspond to samples from two independent experiments. Each circle indicates the bacterial load of an individual flea, and the horizontal lines indicate the median CFU per flea.

tional glucans in *E. coli* and *D. dadantii*. On one hand, *Y. pseudotuberculosis* might produce OPGs under particular conditions or might lack an essential factor for OPG biosynthesis. The latter might be a polyprenyl-phosphate carrier because this conserved molecule has a key role in glycan biosynthesis in several kingdoms, and it has been suggested that UPD-Glc has to be transferred to a polyprenyl-phosphate carrier prior to the assembly of OPGs (55, 56). On the other hand, *Y. pseudotuberculosis* may constitutively accumulate certain ionic and/or nonionic solutes potentially involved in controlling glycosyltransferase activity in response to osmotic stress (57, 58). However, we found that 100-fold overexpression of the *opgGH* operon did not bypass this putative repression. Furthermore, the screening of ~1,000 strains from a Tn5 mutant library of *Y. pseudotuberculosis* for OPG synthesis did not lead to the identification of this type of inhibitor (unpublished data). Hence, the data support the hypothesis whereby *Y. pseudotuberculosis* lacks a functional intermediate that is essential for OPG biosynthesis.

The apparent absence of OPGs in both *Y. pseudotuberculosis* and *Y. pestis* (the latter being naturally devoid of *opgGH*) raises the question of how these species compensate for the lack of OPGs. Suppressor mutations that rescue phenotypes associated with loss of OPGs have been identified in *E. coli*, *D. dadantii*, and members of the *Rhizobiaceae* (35–38, 59–61). All of these mutations affect regulatory systems, including OmpR-EnvZ, RcsCDB, and a phosphodiesterase regulating cyclic di-GMP signaling. However, none of the suppressor mutations completely alleviates the loss of OPGs. Hence, *Y. pseudotuberculosis* and *Y. pestis* may have several suppressor mutations, i.e., a very different regulatory network compared with the above-mentioned bacteria. Consistently, *Y. pestis* has altered Rcs and cyclic di-GMP signaling pathways and does not control *ompF* and *ompC* (involved in bacterial adaptation to osmotic conditions) in the same way as *E. coli* and *D. dadantii* (13, 62, 63). However, whatever applies to *Y. pestis* might not apply to *Y. pseudotuberculosis*, since the latter has a functional Rcs system and does not control its c-di-GMP signaling pathways in the same way as the former. Differences in the membrane composition in *Y. pseudotuberculosis* and *Y. pestis* (relative to *E. coli* and *D. dadantii*) may constitute an alternative and/or additional explanation (64). Indeed, membrane composition has a role in bacterial adaptation to the environment (65, 66). Lastly, *Y. pseudotuberculosis* and *Y. pestis* may produce glucans that are equivalent to OPGs, such as the “free oligosaccharides” described in *Campylobacter jejuni* (67).

In agreement with a previous report on *E. coli* (46), we found that OpgH controls the size of *Y. pseudotuberculosis*. Consistently, *Y. pestis* (which naturally lacks *opgGH*) is smaller than *Y. pseudotuberculosis* but was the same size as its recent ancestor when it expressed *opgGH*. However, *Y. pestis* and *Y. pseudotuberculosis* differed in size when cultured at 37°C but not when cultured at 21°C, presumably because *Y. pestis* has a compensatory mechanism. These results indicate that the shape control mechanism has evolved in *Y. pestis*, potentially providing a selective advantage. Indeed, it has been found that a particular cell shape can maximize the use of available nutrients and promote biofilm formation *in vitro* and virulence (e.g., the ability to escape detection and resist phagocytes and complement) (68–73). However, *opgH* expression did not influence the virulence of *Y. pestis* in mice or its ability to colonize and block fleas. In agreement with the virulence data for

Y. pestis, *Y. pseudotuberculosis* lacking OpgH was able to colonize mice normally.

Lastly, our results suggest an evolutionary scenario in which an ancestral strain of *Yersinia* lost (or did not acquire) a cofactor that was important for OPG biosynthesis. However, *opgGH* was initially retained, presumably because the ability to control bacterial size provides an advantage in the external environment. We suggest that the *opgGH* gene was subsequently lost because the OpgH-dependent size control mechanism was no longer necessary or was replaced by another control mechanism. During this evolutionary process, several events (including regulatory network reprogramming) presumably occurred so as to rescue the essential cellular functions associated with OPGs synthesis and the OpgH-dependent size-control mechanism identified in other bacteria. However, there are other possible explanations for the loss of *opgGH*. Indeed, we cannot rule out the possibility that *Y. pseudotuberculosis* synthesizes OPGs in order to survive under specific conditions that have not been identified in other bacteria. Consistently, genomic analysis indicates that *Y. enterocolitica* (the third human-pathogenic *Yersinia* sp.) and all environmental *Yersinia* species (for which the genomes have been sequenced) have an *opgGH* operon. Hence, *Y. pestis* may also have lost the *opgGH* operon because it no longer encounters conditions under which OPG synthesis is required for survival.

ACKNOWLEDGMENTS

We thank Rodolph Beauvois for his help in screening the mutant library.

This work was funded by the Institut National de la Santé et de la Recherche Médicale, the Institut Pasteur de Lille, the Université Lille Nord de France, the Centre National de la Recherche Scientifique, the Région Nord-Pas de Calais, and the European Regional Development Fund as part of the “Action de recherche concertée d’initiative régionale” program (grant 10090077-Présage 35712 to F.S.).

REFERENCES

- Butler T. 1994. *Yersinia* infections: centennial of the discovery of the plague bacillus. Clin Infect Dis 19:655–661. <http://dx.doi.org/10.1093/cinids/19.4.655>.
- Bacot AW, Martin CJ. 1914. Observations on the mechanism of the transmission of plague by fleas. J Hyg Plague 13(Plague Suppl 3):423–439.
- Sebbane F, Jarrett CO, Gardner D, Long D, Hinnebusch BJ. 2006. Role of the *Yersinia pestis* plasminogen activator in the incidence of distinct septicemic and bubonic forms of flea-borne plague. Proc Natl Acad Sci U S A 103:5526–5530. <http://dx.doi.org/10.1073/pnas.0509544103>.
- Pujol C, Bliska JB. 2005. Turning *Yersinia* pathogenesis outside in: subversion of macrophage function by intracellular yersiniae. Clin Immunol 114:216–226. <http://dx.doi.org/10.1016/j.clim.2004.07.013>.
- Sebbane F, Gardner D, Long D, Gowen BB, Hinnebusch BJ. 2005. Kinetics of disease progression and host response in a rat model of bubonic plague. Am J Pathol 166:1427–1439. [http://dx.doi.org/10.1016/S0002-9440\(10\)62360-7](http://dx.doi.org/10.1016/S0002-9440(10)62360-7).
- Achtman M, Morelli G, Zhu P, Wirth T, Diehl I, Kusecek B, Vogler AJ, Wagner DM, Allender CJ, Easterday WR, Chenal-Francisque V, Worsham P, Thomson NR, Parkhill J, Lindler LE, Carniel E, Keim P. 2004. Microevolution and history of the plague bacillus, *Yersinia pestis*. Proc Natl Acad Sci U S A 101:17837–17842. <http://dx.doi.org/10.1073/pnas.0408026101>.
- Chain PS, Carniel E, Larimer FW, Lamerdin J, Stoutland PO, Regala WM, Georgescu AM, Vergez LM, Land ML, Motin VL, Brubaker RR, Fowler J, Hinnebusch J, Marceau M, Medigue C, Simonet M, Chenal-Francisque V, Souza B, Dacheux D, Elliott JM, Derbise A, Hauser LJ, Garcia E. 2004. Insights into the evolution of *Yersinia pestis* through whole-genome comparison with *Yersinia pseudotuberculosis*. Proc Natl Acad Sci U S A 101:13826–13831. <http://dx.doi.org/10.1073/pnas.0404012101>.
- Hinnebusch BJ, Perry RD, Schwan TG. 1996. Role of the *Yersinia pestis*

- hemin storage (*hms*) locus in the transmission of plague by fleas. *Science* 273:367–370. <http://dx.doi.org/10.1126/science.273.5273.367>.
9. Hinnebusch BJ, Rudolph AE, Cherepanov P, Dixon JE, Schwan TG, Forsberg A. 2002. Role of *Yersinia* murine toxin in survival of *Yersinia pestis* in the midgut of the flea vector. *Science* 296:733–735. <http://dx.doi.org/10.1126/science.1069972>.
 10. Kuttyrev V, Mehig RJ, Motin VL, Pokrovskaya MS, Smirnov GB, Brubaker RR. 1999. Expression of the plague plasminogen activator in *Yersinia pseudotuberculosis* and *Escherichia coli*. *Infect Immun* 67:1359–1367.
 11. Sebbane F, Jarrett C, Gardner D, Long D, Hinnebusch BJ. 2009. The *Yersinia pestis* *caf1M1A1* fimbrial capsule operon promotes transmission by flea bite in a mouse model of bubonic plague. *Infect Immun* 77:1222–1229. <http://dx.doi.org/10.1128/IAI.00950-08>.
 12. Erickson DL, Jarrett CO, Callison JA, Fischer ER, Hinnebusch BJ. 2008. Loss of a biofilm-inhibiting glycosyl hydrolase during the emergence of *Yersinia pestis*. *J Bacteriol* 190:8163–8170. <http://dx.doi.org/10.1128/JB.01181-08>.
 13. Sun YC, Jarrett CO, Bosio CF, Hinnebusch BJ. 2014. Retracing the evolutionary path that led to flea-borne transmission of *Yersinia pestis*. *Cell Host Microbe* 15:578–586. <http://dx.doi.org/10.1016/j.chom.2014.04.003>.
 14. Tang L, Weissborn AC, Kennedy EP. 1997. Domains of *Escherichia coli* acyl carrier protein important for membrane-derived-oligosaccharide biosynthesis. *J Bacteriol* 179:3697–3705.
 15. Miller KJ, Kennedy EP, Reinhold VN. 1986. Osmotic adaptation by gram-negative bacteria: possible role for periplasmic oligosaccharides. *Science* 231:48–51. <http://dx.doi.org/10.1126/science.3941890>.
 16. Beaudoin T, Zhang L, Hinz AJ, Parr CJ, Mah TF. 2012. The biofilm-specific antibiotic resistance gene *ndvB* is important for expression of ethanol oxidation genes in *Pseudomonas aeruginosa* biofilms. *J Bacteriol* 194:3128–3136. <http://dx.doi.org/10.1128/JB.06178-11>.
 17. Bhagwat AA, Jun W, Liu L, Kannan P, Dharne M, Pheh B, Tall BD, Kothary MH, Gross KC, Angle S, Meng J, Smith A. 2009. Osmoregulated periplasmic glucans of *Salmonella enterica* serovar Typhimurium are required for optimal virulence in mice. *Microbiology* 155:229–237. <http://dx.doi.org/10.1099/mic.0.023747-0>.
 18. Roset MS, Ciocchini AE, Ugalde RA, Inon de Iannino N. 2006. The *Brucella abortus* cyclic beta-1,2-glucan virulence factor is substituted with O-ester-linked succinyl residues. *J Bacteriol* 188:5003–5013. <http://dx.doi.org/10.1128/JB.00086-06>.
 19. Salanoubat M, Genin S, Artiguenave F, Gouzy J, Mangenot S, Arlat M, Billault A, Brottier P, Camus JC, Cattolico L, Chandler M, Choise N, Claudel-Renard C, Cunnac S, Demange N, Gaspin C, Lavie M, Moisan A, Robert C, Saurin W, Schiex T, Siguier P, Thebault P, Whalen M, Wincker P, Levy M, Weissenbach J, Boucher CA. 2002. Genome sequence of the plant pathogen *Ralstonia solanacearum*. *Nature* 415:497–502. <http://dx.doi.org/10.1038/415497a>.
 20. Zorreguieta A, Ugalde RA. 1986. Formation in *Rhizobium* and *Agrobacterium* spp. of a 235-kilodalton protein intermediate in beta-D(1-2) glucan synthesis. *J Bacteriol* 167:947–951.
 21. Coge V, Gak E, Puskas A, Kaplan S, Bohin JP. 2002. The *opgGIH* and *opgC* genes of *Rhodobacter sphaeroides* form an operon that controls backbone synthesis and succinylation of osmoregulated periplasmic glucans. *Eur J Biochem* 269:2473–2484. <http://dx.doi.org/10.1046/j.1432-1033.2002.02907.x>.
 22. Minsavage GV, Mudgett MB, Stall RE, Jones JB. 2004. Importance of *opgHXcv* of *Xanthomonas campestris* pv. *vesicatoria* in host-parasite interactions. *Mol Plant Microbe Interact* 17:152–161. <http://dx.doi.org/10.1094/MPMI.2004.17.2.152>.
 23. Bhagwat AA, Mithofer A, Pfeffer PE, Kraus C, Spickers N, Hotchkiss A, Ebel J, Keister DL. 1999. Further studies of the role of cyclic beta-glucans in symbiosis. An NdvC mutant of *Bradyrhizobium japonicum* synthesizes cyclodecakis-(1→3)-beta-glucosyl. *Plant Physiol* 119:1057–1064.
 24. Bontemps-Gallo S, Madec E, Dondey J, Delrue B, Robbe-Masselot C, Vidal O, Prouvost AF, Boussemart G, Bohin JP, Lacroix JM. 2013. Concentration of osmoregulated periplasmic glucans (OPGs) modulates the activation level of the RcsCD RcsB phosphorelay in the phytopathogenic bacteria *Dickeya dadantii*. *Environ Microbiol* 15:881–894. <http://dx.doi.org/10.1111/1462-2920.12054>.
 25. Komaniecka I, Choma A. 2003. Isolation and characterization of periplasmic cyclic beta-glucans of *Azorhizobium caulinodans*. *FEMS Microbiol Lett* 227:263–269. [http://dx.doi.org/10.1016/S0378-1097\(03\)00690-6](http://dx.doi.org/10.1016/S0378-1097(03)00690-6).
 26. Altabe SG, Inon de Iannino N, de Mendoza D, Ugalde RA. 1994. New osmoregulated beta(1-3),beta(1-6) glucosyltransferase(s) in *Azospirillum brasilense*. *J Bacteriol* 176:4890–4898.
 27. Bohin JP. 2000. Osmoregulated periplasmic glucans in Proteobacteria. *FEMS Microbiol Lett* 186:11–19. <http://dx.doi.org/10.1111/j.1574-6968.2000.tb09075.x>.
 28. Lacroix JM, Bohin JP. 2010. Osmoregulated periplasmic glucan polymerization requires constant protein synthesis in *Escherichia coli*. *Curr Microbiol* 61:396–400. <http://dx.doi.org/10.1007/s00284-010-9625-2>.
 29. Therisod H, Weissborn AC, Kennedy EP. 1986. An essential function for acyl carrier protein in the biosynthesis of membrane-derived oligosaccharides of *Escherichia coli*. *Proc Natl Acad Sci U S A* 83:7236–7240. <http://dx.doi.org/10.1073/pnas.83.19.7236>.
 30. Briones G, Inon de Iannino N, Steinberg M, Ugalde RA. 1997. Periplasmic cyclic 1,2-beta-glucan in *Brucella* spp. is not osmoregulated. *Microbiology* 143:1115–1124. <http://dx.doi.org/10.1099/00221287-143-4-1115>.
 31. Talaga P, Fournet B, Bohin JP. 1994. Periplasmic glucans of *Pseudomonas syringae* pv. *syringae*. *J Bacteriol* 176:6538–6544.
 32. Breedveld MW, Miller KJ. 1994. Cyclic beta-glucans of members of the family Rhizobiaceae. *Microbiol Rev* 58:145–161.
 33. Kennedy EP. 1982. Osmotic regulation and the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. *Proc Natl Acad Sci U S A* 79:1092–1095. <http://dx.doi.org/10.1073/pnas.79.4.1092>.
 34. Arellano-Reynoso B, Lapaque N, Salcedo S, Briones G, Ciocchini AE, Ugalde R, Moreno E, Moriyon I, Gorvel JP. 2005. Cyclic beta-1,2-glucan is a *Brucella* virulence factor required for intracellular survival. *Nat Immunol* 6:618–625. <http://dx.doi.org/10.1038/nri1202>.
 35. Bouchart F, Delangle A, Lemoine J, Bohin JP, Lacroix JM. 2007. Proteomic analysis of a non-virulent mutant of the phytopathogenic bacterium *Erwinia chrysanthemi* deficient in osmoregulated periplasmic glucans: change in protein expression is not restricted to the envelope, but affects general metabolism. *Microbiology* 153:760–767. <http://dx.doi.org/10.1099/mic.0.2006/000372-0>.
 36. Kannan P, Dharne M, Smith A, Karns J, Bhagwat AA. 2009. Motility revertants of *opgGH* mutants of *Salmonella enterica* serovar Typhimurium remain defective in mice virulence. *Curr Microbiol* 59:641–645. <http://dx.doi.org/10.1007/s00284-009-9486-8>.
 37. Fineran PC, Williamson NR, Lilley KS, Salmond GP. 2007. Virulence and prodigiosin antibiotic biosynthesis in *Serratia* are regulated pleiotropically by the GGDEF/EAL domain protein, PigX. *J Bacteriol* 189:7653–7662. <http://dx.doi.org/10.1128/JB.00671-07>.
 38. Girgis HS, Liu Y, Ryu WS, Tavaoie S. 2007. A comprehensive genetic characterization of bacterial motility. *PLoS Genet* 3:1644–1660.
 39. Mukhopadhyay P, Williams J, Mills D. 1988. Molecular analysis of a pathogenicity locus in *Pseudomonas syringae* pv. *syringae*. *J Bacteriol* 170:5479–5488.
 40. Mahajan-Miklos S, Tan MW, Rahme LG, Ausubel FM. 1999. Molecular mechanisms of bacterial virulence elucidated using a *Pseudomonas aeruginosa*-*Caenorhabditis elegans* pathogenesis model. *Cell* 96:47–56. [http://dx.doi.org/10.1016/S0092-8674\(00\)80958-7](http://dx.doi.org/10.1016/S0092-8674(00)80958-7).
 41. Banta LM, Bohnie J, Lovejoy SD, Dostal K. 1998. Stability of the *Agrobacterium tumefaciens* VirB10 protein is modulated by growth temperature and periplasmic osmoadaptation. *J Bacteriol* 180:6597–6606.
 42. Mah TF, Pitts B, Pellock B, Walker GC, Stewart PS, O'Toole GA. 2003. A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426:306–310. <http://dx.doi.org/10.1038/nature02122>.
 43. Lapaque N, Forquet F, de Chastellier C, Mishal Z, Jolly G, Moreno E, Moriyon I, Heuser JE, He HT, Gorvel JP. 2006. Characterization of *Brucella abortus* lipopolysaccharide macrodomains as mega rafts. *Cell Microbiol* 8:197–206. <http://dx.doi.org/10.1111/j.1462-5822.2005.00609.x>.
 44. Dylan T, Ielpi L, Stanfield S, Kashyap L, Douglas C, Yanofsky M, Nester E, Helinski DR, Ditta G. 1986. *Rhizobium meliloti* genes required for nodule development are related to chromosomal virulence genes in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci U S A* 83:4403–4407. <http://dx.doi.org/10.1073/pnas.83.12.4403>.
 45. de Iannino NI, Ugalde RA. 1989. Biochemical characterization of avirulent *Agrobacterium tumefaciens* *chvA* mutants: synthesis and excretion of beta-(1-2)glucan. *J Bacteriol* 171:2842–2849.
 46. Hill NS, Buske PJ, Shi Y, Levin PA. 2013. A moonlighting enzyme links *Escherichia coli* cell size with central metabolism. *PLoS Genet* 9:e1003663. <http://dx.doi.org/10.1371/journal.pgen.1003663>.
 47. Young KD. 2007. Bacterial morphology: why have different shapes? *Curr Opin Microbiol* 10:596–600. <http://dx.doi.org/10.1016/j.mib.2007.09.009>.

48. Sebbane F, Jarrett CO, Linkenhoker JR, Hinnebusch BJ. 2004. Evaluation of the role of constitutive isocitrate lyase activity in *Yersinia pestis* infection of the flea vector and mammalian host. *Infect Immun* 72:7334–7337. <http://dx.doi.org/10.1128/IAI.72.12.7334-7337.2004>.
49. Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, Schweizer HP. 2005. A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* 2:443–448. <http://dx.doi.org/10.1038/nmeth765>.
50. Dische Z. 1962. General color reactions. *Methods Carbohydr Chem* 1:478–492.
51. Reboul A, Lemaitre N, Titecat M, Merchez M, Deloison G, Ricard I, Pradel E, Marceau M, Sebbane F. 2014. *Yersinia pestis* requires the 2-component regulatory system *OmpR-EnvZ* to resist innate immunity during the early and late stages of plague. *J Infect Dis* 210:1367–1375. <http://dx.doi.org/10.1093/infdis/jiu274>.
52. Sebbane F, Bury-Mone S, Cailliau K, Browaeys-Poly E, De Reuse H, Simonet M. 2002. The *Yersinia pseudotuberculosis* Yut protein, a new type of urea transporter homologous to eukaryotic channels and functionally interchangeable *in vitro* with the *Helicobacter pylori* UreI protein. *Mol Microbiol* 45:1165–1174. <http://dx.doi.org/10.1046/j.1365-2958.2002.03096.x>.
53. Marolda CL, Valvano MA. 1996. The GalF protein of *Escherichia coli* is not a UDP-glucose pyrophosphorylase but interacts with the GalU protein possibly to regulate cellular levels of UDP-glucose. *Mol Microbiol* 22:827–840. <http://dx.doi.org/10.1046/j.1365-2958.1996.01531.x>.
54. Hinchliffe SJ, Howard SL, Huang YH, Clarke DJ, Wren BW. 2008. The importance of the Rcs phosphorelay in the survival and pathogenesis of the enteropathogenic *Yersinia*. *Microbiology* 154:1117–1131. <http://dx.doi.org/10.1099/mic.0.2007/012534-0>.
55. Weissborn AC, Rumley MK, Kennedy EP. 1991. Biosynthesis of membrane-derived oligosaccharides. Membrane-bound glucosyltransferase system from *Escherichia coli* requires polyprenyl phosphate. *J Biol Chem* 266:8062–8067.
56. Hartley MD, Imperiali B. 2012. At the membrane frontier: a prospectus on the remarkable evolutionary conservation of polyprenols and polyprenyl-phosphates. *Arch Biochem Biophys* 517:83–97. <http://dx.doi.org/10.1016/j.abb.2011.10.018>.
57. Rumley MK, Therisod H, Weissborn AC, Kennedy EP. 1992. Mechanisms of regulation of the biosynthesis of membrane-derived oligosaccharides in *Escherichia coli*. *J Biol Chem* 267:11806–11810.
58. Ingram-Smith C, Miller KJ. 1998. Effects of ionic and osmotic strength on the glucosyltransferase of *Rhizobium meliloti* responsible for cyclic beta-(1,2)-glucan biosynthesis. *Appl Environ Microbiol* 64:1290–1297.
59. Bontemps-Gallo S, Madec E, Lacroix JM. 2014. Inactivation of *pecS* restores the virulence of mutants devoid of osmoregulated periplasmic glucans in the phytopathogenic bacterium *Dickeya dadantii*. *Microbiology* 160:766–777. <http://dx.doi.org/10.1099/mic.0.074484-0>.
60. Dylan T, Nagpal P, Helinski DR, Ditta GS. 1990. Symbiotic pseudoevertants of *Rhizobium meliloti* *ndv* mutants. *J Bacteriol* 172:1409–1417.
61. Nagpal P, Khanuja SP, Stanfield SW. 1992. Suppression of the *ndv* mutant phenotype of *Rhizobium meliloti* by cloned *exo* genes. *Mol Microbiol* 6:479–488. <http://dx.doi.org/10.1111/j.1365-2958.1992.tb01492.x>.
62. Gao H, Zhang Y, Han Y, Yang L, Liu X, Guo Z, Tan Y, Huang X, Zhou D, Yang R. 2011. Phenotypic and transcriptional analysis of the osmotic regulator *OmpR* in *Yersinia pestis*. *BMC Microbiol* 11:39. <http://dx.doi.org/10.1186/1471-2180-11-39>.
63. Sun YC, Hinnebusch BJ, Darby C. 2008. Experimental evidence for negative selection in the evolution of a *Yersinia pestis* pseudogene. *Proc Natl Acad Sci U S A* 105:8097–8101. <http://dx.doi.org/10.1073/pnas.0803525105>.
64. Rebeil R, Ernst RK, Gowen BB, Miller SI, Hinnebusch BJ. 2004. Variation in lipid A structure in the pathogenic *Yersinia*. *Mol Microbiol* 52:1363–1373. <http://dx.doi.org/10.1111/j.1365-2958.2004.04059.x>.
65. Roy H, Dare K, Ibba M. 2009. Adaptation of the bacterial membrane to changing environments using aminoacylated phospholipids. *Mol Microbiol* 71:547–550. <http://dx.doi.org/10.1111/j.1365-2958.2008.06563.x>.
66. Klein G, Lindner B, Brabetz W, Brade H, Raina S. 2009. *Escherichia coli* K-12 suppressor-free mutants lacking early glycosyltransferases and late acyltransferases: minimal lipopolysaccharide structure and induction of envelope stress response. *J Biol Chem* 284:15369–15389. <http://dx.doi.org/10.1074/jbc.M900490200>.
67. Nothaft H, Liu X, McNally DJ, Li J, Szymanski CM. 2009. Study of free oligosaccharides derived from the bacterial N-glycosylation pathway. *Proc Natl Acad Sci U S A* 106:15019–15024. <http://dx.doi.org/10.1073/pnas.0903078106>.
68. Singh SP, Montgomery BL. 2011. Determining cell shape: adaptive regulation of cyanobacterial cellular differentiation and morphology. *Trends Microbiol* 19:278–285. <http://dx.doi.org/10.1016/j.tim.2011.03.001>.
69. Cho H, Jonsson H, Campbell K, Melke P, Williams JW, Jedynak B, Stevens AM, Groisman A, Levchenko A. 2007. Self-organization in high-density bacterial colonies: efficient crowd control. *PLoS Biol* 5:e302. <http://dx.doi.org/10.1371/journal.pbio.0050302>.
70. Chaput C, Ecobichon C, Cayet N, Girardin SE, Werts C, Guadagnini S, Prevost MC, Mengin-Lecreulx D, Labigne A, Boneca IG. 2006. Role of *AmiA* in the morphological transition of *Helicobacter pylori* and in immune escape. *PLoS Pathog* 2:e97. <http://dx.doi.org/10.1371/journal.ppat.0020097>.
71. Weiser JN. 2013. The battle with the host over microbial size. *Curr Opin Microbiol* 16:59–62. <http://dx.doi.org/10.1016/j.mib.2013.01.001>.
72. Justice SS, Hung C, Theriot JA, Fletcher DA, Anderson GG, Footer MJ, Hultgren SJ. 2004. Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. *Proc Natl Acad Sci U S A* 101:1333–1338. <http://dx.doi.org/10.1073/pnas.0308125100>.
73. Dalia AB, Weiser JN. 2011. Minimization of bacterial size allows for complement evasion and is overcome by the agglutinating effect of antibody. *Cell Host Microbe* 10:486–496. <http://dx.doi.org/10.1016/j.chom.2011.09.009>.