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CbrA mediates colicin M resistance in *Escherichia coli* through modification of undecaprenyl-phosphate-linked peptidoglycan precursors

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Running head: CbrA-mediated colicin M resistance in *E. coli*

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**Keywords:** CbrA; bacterial cell wall; peptidoglycan; colicin; reductase; undecaprenyl-phosphate; lipid II; bacterial metabolism.

22

23 **ABSTRACT**

24 Colicin M is an enzymatic bacteriocin produced by some *Escherichia coli* strains which  
25 provokes cell lysis of competitor strains by hydrolysis of the cell-wall peptidoglycan lipid II  
26 precursor. The overexpression of a gene, *cbrA* (*gidS*), was shown to protect *E. coli* cells from  
27 the deleterious effects of this colicin but the underlying resistance mechanism was not  
28 established. We here report that a major structural modification of the undecaprenyl-  
29 phosphate carrier lipid and of its derivatives occurred in membranes of CbrA-overexpressing  
30 cells, which explains the acquisition of resistance towards this bacteriocin. Indeed, a main  
31 fraction of these lipids, including the lipid II peptidoglycan precursor, now display a saturated  
32 isoprene unit at the  $\alpha$ -position, *i.e.* the unit closest to the colicin M cleavage site. Only  
33 unsaturated forms of these lipids were normally detectable in wild-type cells. *In vitro* and *in*  
34 *vivo* assays showed that colicin M did not hydrolyze  $\alpha$ -saturated lipid II, clearly identifying  
35 this substrate modification as the resistance mechanism. These saturated forms of  
36 undecaprenyl-phosphate and lipid II remained substrates of the different enzymes  
37 participating in peptidoglycan biosynthesis and carrier lipid recycling, allowing this colicin  
38 M-resistance mechanism to occur without affecting this essential pathway.

39

40 **IMPORTANCE**

41 Overexpression of the chromosomal *cbrA* gene allows *E. coli* to resist to colicin M (ColM), a  
42 bacteriocin specifically hydrolyzing the lipid II peptidoglycan precursor of targeted cells. This  
43 resistance results from a CbrA-dependent modification of the precursor structure, *i.e.*  
44 reduction of the  $\alpha$ -isoprenyl bond of C<sub>55</sub>-carrier lipid moiety that is proximal to ColM  
45 cleavage site. This modification, observed here for the first time in eubacteria, annihilates the  
46 ColM activity without affecting peptidoglycan biogenesis. These data, which further increase

47 our knowledge on the substrate specificity of this colicin, highlight the capability of *E. coli* to  
48 generate reduced forms of C<sub>55</sub>-carrier lipid and its derivatives. Whether the function of this  
49 modification is only relevant with respect to ColM resistance is now questioned.

50

## 51 INTRODUCTION

52 Colicin M (ColM) is a bacteriocin that certain strains of *Escherichia coli* produce in order  
53 to kill competitor strains of *E. coli* or related species (1). This colicin, which had been known  
54 for a long time to interfere with bacterial cell wall biogenesis (2), was later identified as a  
55 hydrolase (phosphodiesterase) catalyzing the specific degradation of the peptidoglycan lipid II  
56 intermediate precursor undecaprenyl-pyrophospho-MurNAc(-pentapeptide)-GlcNAc (3).  
57 ColM-treated *E. coli* cells indeed accumulated undecaprenol and 1-pyrophospho-MurNAc(-  
58 pentapeptide)-GlcNAc as lipid II degradation products and the resulting depletion of the pool  
59 of lipid II rapidly led to the arrest of peptidoglycan synthesis and ultimately to cell lysis (3).  
60 The crystal structure of ColM was determined (4) and active site amino acid residues were  
61 identified using site-directed mutagenesis experiments (5). Several ColM homologues that are  
62 produced by a restricted number of strains from *Pseudomonas*, *Burkholderia* and  
63 *Pectobacterium* species were subsequently identified and characterized both biochemically  
64 and structurally (6–9).

65 ColM is the only colicin targeting peptidoglycan metabolism as all the other colicins  
66 identified to date exhibit either a membrane pore-forming or a nuclease activity (1,10). Its  
67 mode of action requires several successive essential steps: (i) first, its binding to a specific  
68 receptor, FhuA, present in the outer membrane of susceptible cells (11); (ii) its subsequent  
69 internalization into the periplasmic space via the TonB/ExbB/ExbD translocation machinery  
70 (1,10); (iii) its maturation by the bifunctional periplasmic protein FkpA exhibiting chaperone  
71 and peptidyl-prolyl-*cis-trans* isomerase activities (12), after which ColM can reach and

72 degrade its target, *i.e.* the lipid II molecules present on the outer side of the plasma membrane.  
73 *E. coli* mutants (*fhuA*, *tonB* or *fkpA*) defective in either of these steps are fully resistant to  
74 ColM. Naturally occurring ColM-producing *E. coli* isolates express the ColM gene (*cma*)  
75 together with a *cmi* immunity gene on a plasmid (13). Low-level expression of the Cmi  
76 protein in the periplasm of wild-type cells confers them high-level resistance to ColM (14).  
77 However, the mechanism of action of the Cmi immunity protein still remains unknown. More  
78 recently, the overexpression of an *E. coli* gene named *cbrA* (formerly *yidS*) was shown to  
79 render cells specifically resistant to ColM (15). Based on the sequence similarities it exhibits  
80 with geranylgeranyl reductases (GGR) and other FAD-dependent oxidoreductases, Helbig *et*  
81 *al.* suggested that the CbrA protein might function as a reductase of isoprenoid molecules.  
82 However, these authors did not detect any modification of the pools and structure of  
83 undecaprenyl-phosphate (C<sub>55</sub>-P) derivative lipids in CbrA-overexpressing cells, leading them  
84 to conclude that hydrogenation of peptidoglycan lipid intermediates should not be the  
85 mechanism of CbrA-mediated ColM resistance (15). They also purified the CbrA protein and  
86 showed it contained non-covalently bound FAD, but they failed to demonstrate any enzymatic  
87 activity with this preparation (15). In the present study, we revisited this question and  
88 demonstrated that overexpression of CbrA in *E. coli* cells resulted in the accumulation in the  
89 cell membranes of a new form of carrier lipid displaying a saturated  $\alpha$ -prenyl unit. This  
90 modified dihydro-C<sub>55</sub>-P lipid is shown to be accepted as substrate both *in vitro* and *in vivo* by  
91 the MraY and MurG enzymes, and the  $\alpha$ -saturated form of lipid II thus generated to be  
92 efficiently used by peptidoglycan polymerases. However, contrary to the natural lipid II, this  
93  $\alpha$ -saturated form of lipid II appeared as totally resistant to the hydrolase activity of ColM.

94

## 95 **RESULTS**

96       **Expression of CbrA and colicin M resistance.** The *cbrA* (*yidS*) gene of *E. coli* was  
97 cloned into p*Trc*99A and p*Trc*His30 vectors that allow expression of the protein in wild-type  
98 and N-terminal His-tagged forms, respectively, under the control of the strong IPTG-  
99 inducible *trc* promoter. The resulting plasmids pMLD391 and pMLD682 were shown to  
100 confer high-level resistance to ColM to *E. coli* cells, confirming previous data from Helbig *et*  
101 *al.* (15). Indeed, only faint growth inhibition zones were observed when 5  $\mu$ l of an undiluted  
102 stock of ColM (1 mg/ml) was spotted on 2YT-agar plates seeded with the  
103 BW25113(pMLD391) and BW25113(pMLD682) strains, while clear lysis zones were  
104 observed with the control strain BW25113(p*Trc*99A) for up to a 10<sup>5</sup>-fold dilution of the same  
105 ColM stock (Fig. 1A and C). This protection provided by CbrA was similarly observed when  
106 cells were grown in liquid medium (Fig. 1B). No induction by IPTG was required, showing  
107 that the basal expression of the *cbrA* gene from these plasmids was already sufficient to  
108 confer resistance to the highest concentration of ColM tested here. The fact that both  
109 pMLD391 and pMLD682 plasmids conferred ColM resistance demonstrated that the addition  
110 of an N-terminal His<sub>6</sub>-tag did not affect the protein activity (Fig. 1A and C).

111       **Effect of CbrA overproduction on the pools and structures of C<sub>55</sub>-isoprenoids.** The  
112 prototroph *E. coli* strain FB8 was used as a genetic background for analyzing the effects of  
113 the overexpression of the *cbrA* gene on the pools of C<sub>55</sub>-P derivatives present in cell  
114 membranes. This strain, transformed with either the empty vector p*Trc*99A or the *cbrA*-  
115 expressing pMLD391 plasmid, was grown exponentially in 2YT-ampicillin medium and the  
116 pools of C<sub>55</sub>-isoprenoids were extracted and analyzed by HPLC as described in Materials and  
117 Methods. Fig. 2A shows a typical profile obtained for a control cell extract in which the peak  
118 corresponding to C<sub>55</sub>-P is indicated. The identity of this compound eluted at *ca.* 20 min was  
119 confirmed by comparison with an authentic standard and by MALDI-TOF mass spectrometry  
120 analysis, as described previously (16). Integration of this peak allowed us to determine a pool

121 value of *ca.* 250 nmol/g of cell dry weight for this lipid in FB8 strain (Table 1). Interestingly,  
122 an additional peak was observed when the extract from the *cbrA*-expressing strain was  
123 analyzed in the same conditions, which eluted 2-3 min after the peak of C<sub>55</sub>-P (Fig. 2C). This  
124 compound was collected and its mass spectrometry analysis yielded an *m/z* value of 848.00  
125 for the [M-H]<sup>-</sup> ion, which was by 2 Da higher than that of C<sub>55</sub>-P (calculated *m/z* value,  
126 845.66), suggesting that this new compound differed from C<sub>55</sub>-P by the saturation of one  
127 prenyl unit (Fig. 3B). A sample of commercial authentic dihydro-C<sub>55</sub>-P had the same retention  
128 time in HPLC and showed an *m/z* value of 848.10 (Fig. 3A). That the overexpression of the  
129 *cbrA* gene resulted in the accumulation of a hydrogenated form of C<sub>55</sub>-P in the cell  
130 membranes thus demonstrated the reductase activity of the CbrA protein. Moreover, the  
131 amount of dihydro-C<sub>55</sub>-P significantly increased when *cbrA* gene expression was further  
132 induced by adding 0.5 mM IPTG one hour before the culture was arrested (Table 1). As  
133 earlier reported (16), applying the Kato extraction procedure to these membrane extracts also  
134 allowed us to determine the pools of the C<sub>55</sub>-PP precursor and of its dihydro derivative (Table  
135 1). Our data thus showed that a significant proportion of total C<sub>55</sub>-isoprenoids was present in  
136 their dihydro form in the membranes of CbrA-overexpressing cells, *ca.* 33% and 61% in  
137 absence and presence of IPTG, respectively.

138 We previously reported that the treatment of *E. coli* cells by the lipid II-degrading colicin  
139 M (ColM) resulted in the accumulation of undecaprenol (C<sub>55</sub>-OH) in cell membranes, a  
140 compound that does not normally exist in *E. coli* and could not be reused for recycling the  
141 active form C<sub>55</sub>-P of the carrier lipid (16). We here confirmed that control FB8 cells  
142 accumulated C<sub>55</sub>-OH following treatment by ColM (Fig. 2B and Table 1). The overexpression  
143 of the *cbrA* gene greatly reduced but did not abolish the formation of C<sub>55</sub>-OH resulting from  
144 ColM treatment (Fig. 2D and Table 1). Interestingly, dihydro-C<sub>55</sub>-OH was not detected in  
145 these cells, although dihydro forms of C<sub>55</sub>-P and C<sub>55</sub>-PP were readily detected, suggesting that

146 C<sub>55</sub>-OH was not a substrate of the CbrA reductase. However, these *in vivo* data did not allow  
147 us to conclude on the substrate specificity of this reductase. Indeed, the rapid turnover of the  
148 C<sub>55</sub>-P carrier lipid pool known to occur during peptidoglycan biosynthesis implies that if such  
149 a modification was to concern only C<sub>55</sub>-P, C<sub>55</sub>-PP or lipid II, it would then be quickly spread  
150 and shared by all these lipids *in fine*. Moreover, the absence of dihydro-C<sub>55</sub>-OH in cell  
151 membranes also suggested that if produced, dihydro-lipid II molecules should be resistant to  
152 the ColM hydrolase activity. The latter hypothesis was therefore tested using appropriate *in*  
153 *vitro* assays, as described below.

154 **Localization of the reduced isoprenyl unit.** Which one of the eleven double bonds  
155 present in C<sub>55</sub>-P (Fig. 4) was reduced by CbrA was identified using mild acid hydrolysis  
156 experiments. Indeed, owing to the presence of the allyl phosphate linkage and the ready  
157 formation of an allylic carbocation, this carrier lipid and its derivatives are prone to acid  
158 hydrolysis and to further rearrangements (17,18). As shown in Fig. S1, the C<sub>55</sub>-P lipid  
159 extracted and purified from *E. coli* cell membranes was totally degraded following incubation  
160 in 1 M HCl for 30 min at 100°C, whereas the dihydro-C<sub>55</sub>-P lipid purified from the CbrA-  
161 overexpressing cells perfectly resisted to these mild acid hydrolysis conditions. This  
162 difference of behavior between the C<sub>55</sub>-P allyl derivative and the dihydro-C<sub>55</sub>-P alkyl  
163 derivative allowed us to conclude that the double bond targeted by CbrA was the  $\alpha$ -isoprenyl  
164 unit.

165 **Saturation of lipid II  $\alpha$ -isoprenyl unit abolishes ColM activity.** The above results thus  
166 strongly suggested that the acquired resistance to ColM of the CbrA-overexpressing cells  
167 resulted from the saturation of the  $\alpha$ -isoprenyl unit present in the C<sub>55</sub> lipid moiety of the  
168 peptidoglycan lipid II intermediate, *i.e.* the ColM target. That the reduction of this bond,  
169 which is very close to the ColM cleavage site (Fig. 4), may abolish or at least reduce the  
170 activity of this enzyme was indeed highly conceivable. Evidence for this was provided in



171 different ways. First, we made valuable use of the C<sub>55</sub>-P and dihydro-C<sub>55</sub>-P extracts purified  
172 by HPLC from the *E. coli* membranes (Fig. 2) to generate the corresponding radiolabeled  
173 peptidoglycan lipid I intermediates with purified MraY translocase and UDP-MurNAc-  
174 [<sup>14</sup>C]pentapeptide. We had indeed previously shown that ColM cleaved both the lipid I and  
175 lipid II peptidoglycan intermediates (3) and that MraY could accept reduced as well as  
176 unreduced polyprenyl-phosphates of various sizes as substrates (19,20). As shown in Fig. 5,  
177 lipid I generated from the “natural” unreduced C<sub>55</sub>-P lipid could subsequently be cleaved by  
178 ColM, a reaction releasing 1-pyrophospho-MurNAc-pentapeptide as a product, as reported  
179 earlier (3). However, when dihydro-C<sub>55</sub>-P instead of C<sub>55</sub>-P was used, radiolabeled dihydro-  
180 lipid I was generated but no subsequent cleavage by ColM occurred (Fig. 5).

181 Then, purified radiolabeled lipids II were also synthesized, using this time commercial  
182 sources of C<sub>55</sub>-P and dihydro-C<sub>55</sub>-P, purified MraY and MurG enzymes, UDP-MurNAc-  
183 pentapeptide, and UDP-[<sup>14</sup>C]GlcNAc as the radiolabeled substrate. The data shown in Fig. 6  
184 clearly demonstrated that ColM exhibited its hydrolytic activity only on the natural,  
185 unreduced version of the lipid II, consistent with the preceding observations with lipid I. That  
186 the dihydro-lipid II was not substrate of ColM *in vitro* was thus consistent with the acquired  
187 ColM-resistant phenotype of CbrA-overexpressing cells. It also explained why only  
188 undecaprenol (C<sub>55</sub>-OH), but not dihydro-C<sub>55</sub>-OH, was detectable in membranes from these  
189 cells following ColM treatment.

190 **Dihydro-lipid II sustains peptidoglycan synthesis.** The basal-level expression of CbrA  
191 provided by plasmid pMLD391 in the absence of IPTG was sufficient to confer ColM  
192 resistance without affecting bacterial growth (Fig. 1). This indicated that the ongoing rate of  
193 cell-wall peptidoglycan synthesis might not be significantly affected in these conditions,  
194 thereby suggesting that the dihydro-lipid II molecules thus generated were accepted as  
195 substrate by the peptidoglycan polymerases (glycosyltransferases) acting immediately

196 downstream in this pathway. It also suggested that the C<sub>55</sub>-PP phosphatase activities that play  
197 an essential role in the regeneration (recycling) of the C<sub>55</sub>-P carrier lipid pool could  
198 dephosphorylate and allow recycling of dihydro-C<sub>55</sub>-PP molecules released during the latter  
199 polymerization steps.

200 As multiple peptidoglycan glycosyltransferases and C<sub>55</sub>-PP phosphatases exist in *E. coli*,  
201 whether only some members of these protein families could accept dihydro-substrates was  
202 then questioned and tested using appropriate mutant strains.

203 *E. coli* produces several glycosyltransferases: the three class-A, bifunctional penicillin-  
204 binding proteins PBP1A, PBP1B and PBP1C (which also exhibit transpeptidase activity) and  
205 the monofunctional glycosyltransferase MtgA, which are encoded by the *mrcA*, *mrcB*, *pbpC*  
206 and *mtgA* genes, respectively (21,22). The SEDS-family (shape, elongation, division, and  
207 sporulation) proteins FtsW and RodA were also recently shown to act as peptidoglycan  
208 transglycosylases (23,24). The *mrcA* and *mrcB* genes are individually dispensable but  
209 inactivation of both genes is lethal, PBP1A and PBP1B being two main peptidoglycan  
210 glycosyltransferases in this species. The *mrcA* and *mrcB* single mutants, and the *mrcB pbpC*  
211 *mtgA* triple mutant expressing PBP1A as the sole glycosyltransferase (25), were transformed  
212 by the *cbrA*-expressing pMLD679 plasmid or the pUCP24Nco control vector and the  
213 resulting transformants were then tested for their susceptibility to ColM. This vector  
214 conferring gentamicin resistance was preferentially used here to avoid any effect of ampicillin  
215 on the PBPs activity. As shown in Fig. 7 (panels A and B), the *mrcB* mutant and the triple  
216 mutant appeared slightly more susceptible to ColM than the wild-type and *mrcA* mutant  
217 strains, a finding likely correlated with the lower peptidoglycan content and hyper-  
218 susceptibility to cell-wall targeting agents characterizing PBP1B-deficient strains (26).  
219 However, overexpression of CbrA conferred ColM resistance to all of these strains, without  
220 yielding any apparent toxicity effect (Fig. 7). This result demonstrated that both PBP1A and

221 PBP1B were able to catalyze peptidoglycan polymerization using dihydro-lipid II as  
222 substrate.

223 Similarly, in *E. coli*, four C<sub>55</sub>-PP phosphatases were identified which conjointly participate  
224 in the recycling of C<sub>55</sub>-PP molecules that are released during peptidoglycan polymerization  
225 steps. These integral membrane proteins belong to two protein families: BacA and PAP2  
226 (phosphatidic acid phosphatases of type 2) proteins PgpB, YbjG and LpxT, all of them having  
227 their active site exposed toward the periplasm (27,28). None of them is individually essential  
228 for growth but construction of multiple conditional mutants showed that at least one of the  
229 three proteins BacA, YbjG or PgpB was required and sufficient to sustain cell-wall  
230 peptidoglycan synthesis and ensure cell viability (29). To know whether each of these  
231 different phosphatases can dephosphorylate/recycle dihydro-C<sub>55</sub>-PP, the protection provided  
232 by CbrA against ColM was assessed in mutants expressing only one of them. These deletion  
233 mutants: BW25113  $\Delta$ *pgpB*  $\Delta$ *lpxT*  $\Delta$ *bacA* (YbjG only), BW25113  $\Delta$ *ybjG*  $\Delta$ *lpxT*  $\Delta$ *bacA* (PgpB  
234 only) and BW25113  $\Delta$ *ybjG*  $\Delta$ *lpxT*  $\Delta$ *pgpB* (BacA only), and the parental BW25113 strain,  
235 were here too transformed by the *cbrA*-expressing pMLD679 plasmid or the empty vector  
236 pUCP24Nco, and the susceptibility of transformants to ColM was then determined. As shown  
237 in Fig. 7 (panels C and D), all these mutants appeared significantly protected from ColM  
238 following CbrA overexpression. Interestingly, the strain expressing PgpB as the unique C<sub>55</sub>-  
239 PP phosphatase appeared more susceptible to ColM than the other strains in the assay  
240 conditions used (ColM at 15 ng/ml), suggesting that the recycling of the C<sub>55</sub>-P pool might be  
241 partially slowed down in that strain. These different results showed that the main  
242 peptidoglycan polymerases and C<sub>55</sub>-P-recycling phosphatases tolerated this structural  
243 modification (reduction) of the carrier lipid structure and remained active in *cbrA*-  
244 overexpression conditions.

245 That the CbrA-dependent incorporation of dihydro-intermediates in the C<sub>55</sub>-P carrier lipid  
246 cycle could affect the cell peptidoglycan content and structure was then questioned.  
247 BW25113 cells overexpressing (pMLD679) or not (pUCP24Nco vector) the CbrA protein  
248 were grown exponentially, harvested at the same OD of 1.0, and the peptidoglycan polymer  
249 was extracted and quantitated as previously described (30). The peptidoglycan content of both  
250 strains appeared identical, *ca.* 2800 ± 70 nanomoles (in terms of diaminopimelic acid content  
251 per liter of culture), indicating that the rate of peptidoglycan biosynthesis was not affected in  
252 these conditions of CbrA overexpression, a finding consistent with the demonstrated  
253 capability of peptidoglycan synthetases to utilize dihydro-C<sub>55</sub>-containing substrates. The fine  
254 structure of the latter purified peptidoglycan preparations was then determined using the  
255 classical procedure consisting in the HPLC analysis of fragments (muropeptides) resulting  
256 from the digestion of this polymer by a specific muramidase (mutanolysin) (31). Here too, no  
257 significant change in the pattern of muropeptides and in the overall ratio of dimers versus  
258 monomers (*i.e.*, the peptidoglycan crosslinking index) was observed whether the CbrA protein  
259 was overexpressed or not (Fig. S2).

260 The impact of CbrA overexpression on the cell susceptibility to bacitracin was also tested.  
261 This antibiotic is known to act by tightly binding to the pyrophosphoryl moiety of C<sub>55</sub>-PP, the  
262 sequestration of this lipid preventing the recycling of the C<sub>55</sub>-P carrier lipid and leading to an  
263 arrest of peptidoglycan biosynthesis. We thus analyzed whether the reduction of the α-  
264 isoprenyl unit that is proximal to the pyrophosphoryl group in this lipid could modify the cell  
265 susceptibility to this antibiotic. As shown in Fig. S3, only a slight decrease of the cell  
266 susceptibility to bacitracin was observed following CbrA overexpression, suggesting that  
267 dihydro-C<sub>55</sub>-PP remains a target for this antibiotic. However, the susceptibility of *E. coli* and  
268 other Gram-negative species to this antibiotic being intrinsically very low, due to the presence

269 of the outer membrane, further *in vitro* analyses would be required to estimate the real impact  
270 this modification has on the affinity of bacitracin for the C<sub>55</sub>-PP lipid.

271 **Site-directed mutagenesis of CbrA.** As reported by Helbig *et al.* (15), the CbrA protein  
272 exhibited sequence similarities with geranylgeranyl reductases and other FAD-dependent  
273 oxidoreductases of diverse functions belonging to the *para*-hydroxybenzoate hydroxylase  
274 (PHBH) superfamily. Sequence alignments, together with the elucidation of the crystal  
275 structures of two archeal members of this family, a geranylgeranyl reductase (GGR) from  
276 *Sulfolobus acidocaldarius* (32) and a digeranylgeranyl glycerophospholipid reductase  
277 (DGGR) from *Thermoplasma acidophilum* (33), both in complex with FAD, allowed the  
278 identification of several conserved amino acid residues and motifs likely involved in the  
279 binding of FAD and substrates or in catalysis. In particular, a characteristic YxWxFP motif  
280 located within the catalytic domain was highlighted in these studies, the aromatic residues of  
281 which pointing to the FAD and being suggested to maintain the lipid substrate in the  
282 appropriate position for reduction (residues 215-220 and 209-214 of *S. acidocaldarius* and *T.*  
283 *acidophilum* protein sequences, respectively) (Fig. 8). A widely conserved cysteine residue,  
284 Cys47 and Cys44 in the latter Sa\_GGR and Ta\_GGR proteins, respectively, was also shown  
285 to play an essential role in catalysis (32,33). This residue was indeed present in Ec\_CbrA  
286 (Cys46) but the YxWxFP motif appeared less conserved as only two of the aromatic residues,  
287 namely Tyr202 and Trp204, were found in the CbrA protein sequence (Fig. 8). Site-directed  
288 mutagenesis of these residues, and also of a proximal conserved Lys208 residue, was  
289 performed on the CbrA-expressing plasmid pMLD682 and the resulting variants were tested  
290 for their capability to protect *E. coli* cells against ColM. As shown in Fig. 1A and C, the  
291 replacement of either of the Cys46, Tyr202, Trp204 and Lys208 residues by an alanine  
292 strongly affected the CbrA activity, as judged by the loss of viability observed for these  
293 clones at ColM concentrations that normally did not affect growth of wild-type CbrA-

294 expressing cells. Indeed, the susceptibility to ColM of transformants overexpressing the  
295 Cys46Ala and Trp204Ala CbrA mutants was similar to that of the BW25113 host strain and a  
296 partial protection against this colicin was provided by the Tyr202Ala mutant and to a lesser  
297 extent by the Lys208Ala mutant, suggesting that the reductase activity of the two latter  
298 proteins was not totally abolished. These results, which demonstrated that the catalytic  
299 activity of CbrA was required to confer immunity against ColM, confirmed the essential role  
300 played by these different conserved residues in the reaction mechanism of this reductase, as  
301 previously reported for some members of this protein superfamily (32,33).

### 302 **Purification and enzymatic assays of the CbrA protein.**

303 A purification of the CbrA protein was performed in order to biochemically characterize  
304 this protein and identify its substrate among prenol derivatives. The N-terminally His<sub>6</sub>-tagged  
305 CbrA protein was successfully overproduced in BW25113(pMLD682) cells (Fig. S4) and  
306 shown to be almost exclusively recovered in the membrane fraction following cell disruption,  
307 as already experienced by Helbig *et al.* (15). Contrary to the control, the CbrA-containing  
308 membrane extract was yellowish, likely indicating the presence of oxidized FAD cofactor.  
309 The CbrA protein mainly remained in the pellet fraction following solubilization of  
310 membranes by 4% of *N*-dodecyl- $\beta$ -D-maltopyranoside (DDM) detergent. However,  
311 significant amounts were recovered in the supernatant fraction as the CbrA protein was  
312 subsequently successfully purified from the solubilized membrane fraction, as shown in Fig.  
313 S4. Of note, the final purified sample (yield: *ca.* 0.2 mg of protein per liter of culture) had  
314 apparently lost the oxidized FAD cofactor, as judged by the lack of typical absorption  
315 spectrum.

316 The total membrane extracts and the purified CbrA protein were used for reductase  
317 enzymatic assays as described in Material and Methods. The C<sub>55</sub>-PP and C<sub>55</sub>-P lipids were  
318 extensively incubated with CbrA samples in the presence of FAD and either NADPH, NADH

319 or sodium dithionite as hydrogen donors. HPLC analyses of these reaction mixtures  
320 performed as described in Material and Methods did not reveal any significant conversion of  
321 these lipids nor change of their hydrogenation status (data not shown). The lipid II  
322 peptidoglycan precursor was also tested as CbrA substrate. In that case, radiolabeled lipid II  
323 was incubated overnight with CbrA samples in the presence of FAD and the different  
324 hydrogen donors, and a treatment by ColM was subsequently performed to assess the  
325 conversion of lipid II to a ColM-resistant, dihydro-lipid II product. However, no difference in  
326 the extent of lipid II hydrolysis by ColM was observed between the control and CbrA-treated  
327 lipid II (data not shown), indicating that the  $\alpha$ -isoprenyl bond of this substrate had not been  
328 reduced in these assay conditions.

329 **Toxicity of CbrA overproduction.** As mentioned above, basal expression of the *cbrA*  
330 gene from *pTrc*-derivative plasmids was sufficient, *i.e.* did not need induction by IPTG, to  
331 allow cell protection against ColM. Whether an increased production of CbrA could further  
332 improve this protection was then tested. In fact, the addition of IPTG at a 100  $\mu$ M to 1mM  
333 concentration in the growth medium turned out to be toxic for cell growth. Interestingly, this  
334 toxicity was only detected with the wild-type CbrA protein but not with the mutants whose  
335 capability to protect cells against ColM was abolished or dramatically reduced (Fig. S5).  
336 Indeed, addition of IPTG decreased by a 5 to 6 log factor the number of colonies observed  
337 after overnight growth in the case of the BW25113(pMLD682) strain expressing the wild-  
338 type CbrA protein. No significant loss of viability was observed in these conditions for cells  
339 carrying the pMLD690, pMLD688 and pMLD692 plasmids expressing the Tyr202Ala,  
340 Trp204Ala and Lys208Ala CbrA variants, respectively (Fig. S5). Similar amounts of these  
341 different proteins were detected in cell membrane extracts following overnight growth of the  
342 latter strains in the presence of 1 mM IPTG (Fig. S6), showing that the decreased toxicity of  
343 the four mutant proteins did not result from differences in the expression and/or stability of

344 these proteins. The correlation thus highlighted between the toxicity and the protein activity  
345 showed that this was not simply the overproduction of the CbrA protein but rather the  
346 increase of its specific enzymatic (reductase) activity in the cell content that was responsible  
347 for the observed loss of cell viability.

348 **ColM resistance conferred by Cmi is not CbrA-dependent.** *E. coli* strains are protected  
349 from the colicins they produce via the concomitant expression of an immunity protein (1),  
350 which is named Cmi (or ImM) in the case of ColM. The 3D crystal structure of the Cmi  
351 protein was earlier determined and amino acid residues that are important for its activity were  
352 identified (14). However, by which mechanism Cmi confers ColM resistance remains  
353 unknown. It was thus tempting to speculate that both Cmi and CbrA proteins could mediate  
354 ColM resistance by the same mechanism, *i.e.*, the reduction of the lipid II C<sub>55</sub> moiety.  
355 Whether Cmi could itself be a reductase or may indirectly modulate the expression or activity  
356 of the CbrA protein was then tested. First, an analysis of the pools of C<sub>55</sub>-lipids in membranes  
357 of Cmi-expressing, ColM-resistant cells (carrying the pMLD232 plasmid) did not reveal the  
358 presence of dihydro forms of these lipids (Fig. 2E). Second, we showed that Cmi expression  
359 protected *cbrA* mutant cells as efficiently as the wild-type cells against ColM (Fig. S7),  
360 thereby demonstrating that the Cmi activity did not rely on CbrA and that the Cmi- and CbrA-  
361 mediated resistance mechanisms were unrelated.

362

## 363 **DISCUSSION**

364 The inactivation of either of the genes required for the reception on the cell surface, the  
365 import in the periplasm, or the maturation of ColM confers resistance against this colicin to *E.*  
366 *coli* cells (10,11). Overexpressing the *fhuA* receptor and *tonB/exbBD* import machinery genes  
367 was earlier shown to improve the uptake of ColM and thus to further increase the cell  
368 susceptibility to this colicin (34). To the best of our knowledge, the *cbrA* (*gidS*) gene was to



369 date the only example of a gene whose overexpression resulted in ColM resistance. We here  
370 demonstrated that the CbrA-mediated resistance phenotype resulted from a modification of  
371 structure of the lipid II target that immunized it against the hydrolase activity of this colicin.  
372 This structural modification ultimately shared by all the C<sub>55</sub>-P-derived lipid intermediates  
373 present in the membrane (Fig. 4) was tolerated by the main enzymes involved in  
374 peptidoglycan biosynthesis and C<sub>55</sub>-P carrier lipid recycling, thus allowing ColM resistance to  
375 occur without affecting the cell viability.

376 Helbig *et al.* (15) earlier failed to demonstrate an enzymatic (reductase) activity of CbrA,  
377 using the cell-extracted mixture of undecaprenyl derivatives as substrates, and our here  
378 described attempts were unfortunately not more successful. Indeed, the CbrA protein was  
379 efficiently overproduced and purified in His-tagged form (Fig. S4) but none of the potential  
380 substrates we tested (C<sub>55</sub>-PP, C<sub>55</sub>-P, lipid II) appeared to be reduced by this enzyme in the *in*  
381 *vitro* assay conditions used. Maybe the functional stability of this membrane protein was only  
382 conserved in a particular membrane environment, the recognition of its substrates was  
383 affected by the presence of the detergent or the *in vitro* reconstitution of CbrA activity failed  
384 because this enzyme is requiring a functional partner. This also raised the question of the  
385 identity of the natural substrate(s) and of the physiological role of this enzyme. The detection  
386 of dihydro forms of C<sub>55</sub>-P, C<sub>55</sub>-PP and lipid II in the membranes of CbrA-overexpressing cells  
387 did not mean that all of these lipids were substrates of CbrA. Indeed, the reduction of either of  
388 them was expected to be rapidly propagated to the others, due to the functioning of the C<sub>55</sub>-P  
389 carrier lipid cycle, C<sub>55</sub>-P being used by MraY and MurG enzymes to synthesize lipid II and  
390 the C<sub>55</sub>-PP released from lipid II by transglycosylases being then recycled back to C<sub>55</sub>-P by  
391 the C<sub>55</sub>-PP phosphatases. The finding that C<sub>55</sub>-OH but not dihydro-C<sub>55</sub>-OH accumulated in  
392 membranes of CbrA-overexpressing cells following treatment with ColM suggested that C<sub>55</sub>-  
393 OH was not a substrate of CbrA. However, considering that C<sub>55</sub>-OH is released from lipid II

394 by ColM on the outer side of the inner membrane and that the catalytic site of CbrA is likely  
395 oriented towards the cytoplasmic side of this membrane, the absence of dihydro-C<sub>55</sub>-OH  
396 could simply reflect an incapacity of C<sub>55</sub>-OH, contrary to C<sub>55</sub>-P, to be translocated (flipped)  
397 back to the cytoplasm.

398 To date, the capability to reduce polyprenyl substrates *in vitro* or *in vivo* was confirmed for  
399 a limited number of putative geranylgeranyl reductases (GGR) and related enzymes, most of  
400 them originating from archaeal species (35). Some reductases were specific of pyrophosphate  
401 substrates but other accepted both alcohol and pyrophosphate forms of their isoprenoid  
402 (farnesyl, geranylgeranyl...) substrate and could saturate only one or several of the double  
403 bonds present in these molecules. In some cases, the presence of the pyrophosphate group was  
404 shown to prevent reduction of the  $\alpha$ -prenyl group (35). These enzymes are expected to use  
405 NADPH/NADH or ferredoxin as electron donor, as demonstrated for the *T. acidophilum* (33)  
406 and *Methanosarcina acetivorans* (36) GGR, respectively, but as this is the case for the *S.*  
407 *acidocaldarius* GGR (32), the source of electrons often remains unknown.

408 Whether the here demonstrated reductase activity of CbrA towards lipid II and/or the lipid  
409 carrier reflects its specific physiological role, or is fortuitous and only observable when this  
410 protein is overproduced to unnatural levels, remains unclear. Helbig and coworkers earlier  
411 showed that the expression of the *E. coli cbrA* gene was under the control of the CreBC two-  
412 component regulatory system (15). An 8-fold increase of transcription of this gene (as judged  
413 by using chromosomal *lacZ* fusions) was indeed detected following a shift from rich medium  
414 (LB) to glucose-M9 minimal medium, which conferred partial resistance against ColM.  
415 However, no difference in the susceptibility to ColM was observed between the wild-type and  
416 *cbrA* and *creBC* mutant cells when these strains were grown in rich medium (15). As shown  
417 here, no dihydro-C<sub>55</sub>-P was detected in membrane extracts of the wild-type strain, confirming  
418 that basal expression of CbrA does not provide any significant protection against ColM. That

419 the primary function of this reductase is to protect susceptible *E. coli* strains against ColM-  
420 producing strains seems unlikely. One would rather imagine that the main substrate of this  
421 enzyme still remains to be identified and that the here observed modification of lipid II  
422 structure represents a side-activity exhibited under particular “non-physiological”  
423 circumstances, *i.e.* when this protein is significantly overproduced. Interestingly in this  
424 respect, *cbrA*-overexpressing cells appeared more resistant to osmotic shock than wild-type  
425 cells (15), which made Helbig and coworkers hypothesize that CbrA may impact in some way  
426 the structure of the cell envelope and possibly the outer membrane. We here observed that the  
427 high-level overproduction of the wild-type CbrA protein was toxic for *E. coli* cells, no  
428 overnight growth being detected on plates following IPTG-induction of gene expression from  
429 the *pTrc*-derivative plasmids. However, this toxic effect was not observed with the Cys46Ala  
430 variant and other inactive mutants generated in the present work, demonstrating that the CbrA  
431 enzyme activity, and not simply the protein overproduction, accounted for the observed  
432 phenomena. Whether this results from a more extensive reduction of C<sub>55</sub>-P derivative lipid  
433 intermediates in membranes or the modification of other putative substrates remains to be  
434 elucidated. The fact that the expression of the chromosomal *cbrA* gene is only induced under  
435 stress conditions would suggest a potential implication of these reduced lipids in adaptation to  
436 harsh conditions.

437 Polyprenyl phosphate lipids allow the translocation of sugars/glycans across cell  
438 membranes in all kingdoms of life. They are required for the synthesis of various bacterial  
439 cell-wall polysaccharides (peptidoglycan, teichoic acids, O-antigen...), as well as for protein  
440 glycosylation in eukaryotic, archaea and some bacterial species (37). In bacteria, interfering  
441 with the synthesis (or recycling) of the C<sub>55</sub>-P carrier lipid results in the arrest of peptidoglycan  
442 synthesis and ultimately in cell lysis. This essential carrier lipid and its metabolism thus  
443 represent valuable potential targets in the search of new antibacterial agents (38,39).

444 However, such antibiotics should be highly specific and not toxic for mammal cells,  
445 considering that congenital protein glycosylation disorders are known to lead to severe  
446 malformations and developmental retardations with dramatic outcomes in human (40).  
447 Interestingly in this respect, the structure of the lipid carrier varies to some extent in living  
448 organisms, both in the carbon chain length (mainly C<sub>55</sub> in bacteria, up to C<sub>100</sub> in eukaryotes),  
449 its stereochemistry (*cis/trans* configuration of double bonds), and the hydrogenation status of  
450 the terminal isoprenyl unit bound to the phosphate group (37). This  $\alpha$ -isoprenyl unit remains  
451 unsaturated in bacteria but appears saturated in eukaryotes and archaea, implying the  
452 occurrence of an additional reduction step leading to the so-called dolichyl phosphate carrier  
453 lipid in the latter species. The latter reaction was shown to be catalyzed, at least partially, by  
454 the steroid 5 $\alpha$ -reductase type 3 (SDR5A3) in human (41), a protein that does not exhibit any  
455 significant sequence homology with CbrA (data not shown). The potential utilization of ColM  
456 and its different homologs as an alternative to antibiotics for combating multidrug-resistant  
457 pathogenic species was previously envisaged (42). The present demonstration that ColM  
458 could specifically cleave bacterial lipid II peptidoglycan precursors carrying an unsaturated  
459 C<sub>55</sub> moiety while preserving dihydro-lipid II and thus dolichyl phosphate-linked glycans from  
460 the human host thus reinforces the interest for this class of bacteriocin and supports their  
461 potential use as antibacterial agents.

462

## 463 **MATERIALS AND METHODS**

464 **Bacterial strains, plasmids and growth conditions.** The *E. coli* strain DH5 $\alpha$  (Bethesda  
465 Research Laboratories) was used as the host for propagation of plasmids and the prototroph  
466 FB8 strain was earlier described (43). The wild-type BW25113 strain was obtained from B.  
467 Wanner and the derivative *cbrA* mutant strain (JW5631) was from the Keio collection (44).  
468 Other mutant strains carrying single or multiple deletions of the PBP1A (*mrcA*), PBP1B

469 (*mrcB*), PBP1C (*pbpC*) and MtgA (*mtgA*) genes were previously described (25), as well as  
470 strains carrying single or multiple deletions of the C<sub>55</sub>-PP phosphatase genes (*pgpB*, *ybjG*,  
471 *lpxT* and *bacA*) (27,29,45). The pTrc99A plasmid was from Amersham Biosciences and the  
472 pTrcHis30 (46) and pUCP24Nco (47) plasmid vectors were earlier described. The pMLD232  
473 plasmid used for expression of ColM immunity protein Cmi was previously described (14).  
474 Unless otherwise noted, cells were grown in 2YT medium (48) at 37°C. Ampicillin,  
475 kanamycin, chloramphenicol and gentamicin were used at 100, 50, 25 and 10 µg/ml,  
476 respectively. Growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer.

477 **General DNA techniques and *E. coli* cell transformation.** Gene amplification (PCR)  
478 was performed with a Thermocycler 60 apparatus (Bio-Med) and the Expand-Fidelity  
479 polymerase (Roche) and the resulting fragments were purified using Wizard purification kits  
480 (Promega). Standard procedures for gel electrophoresis, gene cloning and plasmid purification  
481 were used (49). Site-directed mutagenesis of CbrA enzyme residues was performed directly  
482 on *cbrA*-expressing plasmids by using the “Quikchange II site-directed mutagenesis kit” from  
483 Agilent Technologies and the oligonucleotides shown in Table S1. Transformation of *E. coli*  
484 cells by plasmids was performed as described earlier by Dagert and Ehrlich (50) or by  
485 electroporation.

486 **Construction of expression plasmids.** Plasmids allowing overexpression of the CbrA  
487 protein were constructed as follows. The *cbrA* gene was amplified by PCR from the  
488 chromosome of *E. coli* strain DH5α using PCR primers Cbra-1 and Cbra-2 that incorporated  
489 NcoI and HindIII restriction sites at the 5' and 3' extremities of the gene, respectively (Table  
490 S1). The resulting DNA fragment digested by these enzymes was inserted between the same  
491 sites of the pTrc99A vector, generating the plasmid pMLD391 that allows expression of the  
492 wild-type *cbrA* gene under control of the strong isopropyl-β-D-thiogalactopyranoside (IPTG)-  
493 inducible *trc* promoter. For construction of the pMLD682 plasmid allowing expression of

494 CbrA with a N-terminal His<sub>6</sub>-tag, the gene was amplified using the Cbra-2 and Cbra-3  
495 oligonucleotides (Table S1) and the resulting fragment was cleaved by BamHI and HindIII  
496 and then inserted between the same sites of the p*Trc*His30 vector. The *cbrA* gene-containing  
497 NcoI-HindIII fragment from pMLD391 was also cloned between the same sites of the  
498 pUCP24Nco vector (47), yielding the pMLD679 plasmid (gentamicin resistance). The  
499 pMLD684, pMLD690, pMLD688 and pMLD692 plasmids allowing expression of Cys46Ala,  
500 Tyr202Ala, Trp204Ala and Lys208Ala mutated CbrA proteins, respectively, were generated  
501 by site-directed mutagenesis of the pMLD682 plasmid. The sequence of cloned inserts was  
502 verified by DNA sequencing (Eurofins-MWG).

503 **Preparation of crude extracts, purification of CbrA and enzymatic assays.** *E. coli*  
504 BW25113 cells carrying either the p*Trc*His30 vector as a control or the pMLD682 plasmid  
505 were grown at 37°C in 2YT-ampicillin medium (1-liter cultures). When the optical density of  
506 the culture reached 0.8, IPTG was added at a final concentration of 1 mM and growth was  
507 continued overnight at 22°C. Cells were harvested and washed with 40 ml of cold 20 mM  
508 Tris-HCl buffer (pH 7.4) containing 150 mM NaCl (buffer A). The cell pellet was suspended  
509 in 6 ml of the same buffer and cells were disrupted by sonication in the cold (Bioblock  
510 Vibracell sonicator, model 72412). The resulting suspension was centrifuged at 4°C for 30  
511 min at 200,000 × *g* in a TL100 Beckman centrifuge and the pellet consisting of membranes  
512 and associated proteins (including CbrA) was recovered (total membrane extract). The latter  
513 extract was subjected to solubilization by resuspension in 20 ml of buffer A containing 4%  
514 DDM. This mixture was incubated for 4 h at 4°C with shaking and then centrifuged as  
515 described above to separate non-solubilized proteins (sediment) and solubilized proteins  
516 (supernatant). The supernatant obtained from CbrA-overexpressing BW25113(pMLD682)  
517 cells was used for the purification of the N-terminally His<sub>6</sub>-tagged CbrA protein. It was  
518 incubated for 1 h at 4°C with nickel-nitrilotriacetate (Ni<sup>2+</sup>-NTA) agarose pre-equilibrated in

519 buffer A containing 10 mM imidazole. Then, the polymer was washed extensively with buffer  
520 A containing 0.2% DDM and 10 mM imidazole and with buffer A containing 0.2% DDM and  
521 50 mM imidazole, and elution of proteins was obtained with buffer A containing 0.2% DDM  
522 and 200 mM imidazole. CbrA-containing fractions were dialyzed overnight against 100  
523 volumes of buffer A containing 0.05% DDM. SDS-PAGE analysis of proteins was performed  
524 as described by Laemmli and Favre (51) and protein concentrations were determined with a  
525 NanoDrop apparatus (Thermo Scientific).

526 Enzymatic (reductase) CbrA assays using C<sub>55</sub>-PP or C<sub>55</sub>-P as substrate were performed as  
527 follows. The reaction mixture (100 µl) contained 0.5 mM of lipid substrate, 20 mM Tris-HCl,  
528 pH 7.4, 150 mM NaCl, 0.2% DDM, 100 µM FAD, and 100 mM of either NADPH, NADH or  
529 sodium dithionite. Total membrane extracts (5 µl) or the purified CbrA protein (30 µl, about 3  
530 µg of protein) were added to the reaction mixture, which was then incubated overnight at  
531 30°C with shaking. The lipids were recovered by extraction with butanol, dried under  
532 vacuum, eventually submitted to Kato's procedure (C<sub>55</sub>-PP samples), and analyzed by HPLC  
533 as described in this report and previously (16) to determine their status of hydrogenation.

534 Enzymatic assays using lipid II as substrate were performed in a reaction mixture (20 µl)  
535 containing 12 µM [<sup>14</sup>C]radiolabeled lipid II in the buffer used for ColM enzymatic activity  
536 assay (see below) supplemented by 1 mM NADPH or sodium dithionite. 2 µl of CbrA-  
537 containing membrane extracts or purified CbrA protein were added to the reaction mixture,  
538 which was incubated overnight at 30°C. Then, 2 µl of purified ColM was added and the latter  
539 mixture was incubated for 1 h at 37°C and the extent of lipid II hydrolysis was evaluated by  
540 thin-layer chromatography (TLC) on LK6D silica gel plates (Whatman), using 1-  
541 propanol/ammonium hydroxide/water (6/3/1, v/v) as the mobile phase. Radioactive spots  
542 were located and quantified with a radioactivity scanner (Rita Star, Raytest  
543 Isotopenmeßgeräte GmbH, Straubenhardt, Germany).

544 **ColM enzymatic activity assay.** The hydrolase activity of ColM was routinely tested in a  
545 reaction mixture (10  $\mu$ l) containing 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl<sub>2</sub>,  
546 10 mM  $\beta$ -mercaptoethanol, 12  $\mu$ M [<sup>14</sup>C]radiolabeled lipid II (140 Bq), 0.2% DDM, and  
547 purified ColM (5  $\mu$ l of appropriate dilution in buffer A). Some assays used lipid I instead of  
548 lipid II as the substrate. In that case, radiolabeled lipid I was generated by using purified  
549 MraY enzyme, UDP-MurNAc-[<sup>14</sup>C]pentapeptide, and either C<sub>55</sub>-P or dihydro-C<sub>55</sub>-P as lipid  
550 substrate. Reaction mixtures were incubated for 1 h at 37°C with shaking (Thermomixer,  
551 Eppendorf) and reactions were stopped by heating at 100°C for 1 min. These mixtures were  
552 analyzed by TLC and the radioactive spots were detected and quantified as described above.

553 **ColM cytotoxicity assays.** The cytotoxic activity of ColM was tested on 2YT-agar plates  
554 overlaid with 3 ml of soft nutrient agar containing *ca.* 10<sup>8</sup> cells of the *E. coli* strains to be  
555 tested. Serial dilutions of pure ColM-containing samples were prepared in buffer A and 5  $\mu$ l  
556 aliquots were spotted on the overlay. Plates were incubated for 24 h at 37°C and the  
557 sensitivity of strains and efficiency of ColM was judged from the clear zones of growth  
558 inhibition observed at the spot position. Alternatively, ColM was incorporated in the 2YT-  
559 agar medium and 5  $\mu$ l of serial dilutions of cultures of the *E. coli* strains to be tested were  
560 spotted on the plates. ColM cytotoxicity was also tested in 2YT liquid medium by adding  
561 ColM at different concentrations to exponentially growing cultures of *E. coli*.

562 **Extraction and purification of C<sub>55</sub>-P and its derivatives from *E. coli* membranes.** Cell  
563 pellets harvested from 100-ml cultures of the different strains to be tested were treated  
564 essentially as described previously (16). One half of the cell pellet was submitted to the  
565 procedure of Kato *et al.* (52) that allows conversion of C<sub>55</sub>-PP into C<sub>55</sub>-P, and lipids were in  
566 all cases extracted according to Bligh and Dyer (53) with minor modifications (16). Extracts  
567 were analyzed by HPLC as earlier described (16) and injections of standard commercial lipids  
568 in the same conditions allowed the calibration and the quantification of these lipids in cell



569 extracts. Mild acid hydrolysis experiments were performed to allow identification of the  
570 reduced bond in the dihydro-C<sub>55</sub>-P lipid extracted from *cbrA*-overexpressing cells. Samples of  
571 C<sub>55</sub>-P and dihydro-C<sub>55</sub>-P lipids of commercial source or purified from *E. coli* cells were  
572 treated or not with 1 M HCl for 30 min at 100°C and the resulting mixtures were analyzed by  
573 HPLC as described above.

574 **Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass**  
575 **spectrometry analysis of lipids.** MALDI-TOF mass spectra were recorded in the linear mode  
576 with delayed extraction on a PerSeptive Voyager-DE STR instrument (Applied Biosystems)  
577 equipped with a 337-nm laser. Aliquots were dissolved in methanol-2-propanol 1:1 (v/v),  
578 mixed with two volumes of 100 mM terthiophene in ethyl acetate, and then deposited (1 µl)  
579 on sample plates. Spectra were recorded in the reflectron mode at an acceleration voltage of -  
580 20 kV and an extraction delay time of 200 ns. A mixture of peptidoglycan precursors (UDP-  
581 MurNAc, UDP-MurNAc-dipeptide and UDP-MurNAc-pentapeptide) was used as an external  
582 calibrant.

583 **Extraction and quantification of peptidoglycan.** BW25113 cells (0.8-liter cultures)  
584 carrying either the *cbrA*-expressing plasmid pMLD679 or the empty vector pUCP24Nco were  
585 grown exponentially at 37°C in 2YT-gentamicin medium. At an OD<sub>600nm</sub> of 1.0, bacteria were  
586 harvested in the cold, suspended in a few ml of 0.9% NaCl solution, and then injected under  
587 vigorous stirring in 30 ml of a hot (95-100°C) aqueous 4% SDS solution. After 1 h of this  
588 treatment, suspensions were kept overnight at room temperature and then centrifuged for 30  
589 min at 200,000 × *g*. The resulting pellets containing the peptidoglycan polymer were washed  
590 several times with water and after final resuspension in 3 ml of water, aliquots were  
591 hydrolyzed (16 h at 95°C in 6 M HCl) and analyzed with a Hitachi model 8800 amino acid  
592 analyzer (ScienceTec). The cell peptidoglycan content was expressed in terms of its  
593 characteristic components (diaminopimelic acid, muramic acid), as previously reported (30).

594 Its fine structure was determined by using the classical procedure of Glauner consisting in the  
595 analysis of fragments (muropeptides) released following digestion of this polymer by a  
596 muramidase (31). The purified peptidoglycan preparations were digested by mutanolysin and  
597 muropeptides were reduced with sodium borohydride and then separated by HPLC on a 3- $\mu$ m  
598 ODS-Hypersil column (4.6  $\times$  250 mm, Thermo Scientific). A gradient of methanol (from 0 to  
599 25% in 90 min) in 50 mM sodium phosphate, pH 4.5, was used for elution, at a flow rate of  
600 0.5 ml/min. Peaks of muropeptides were detected at 207 nm.

601 **Chemicals.** Undecaprenyl-pyrophosphate (C<sub>55</sub>-PP), undecaprenyl-phosphate (C<sub>55</sub>-P),  
602 undecaprenol (C<sub>55</sub>-OH), and their dihydro derivatives were provided by the Institute of  
603 Biochemistry and Biophysics of the Polish Academy of Sciences. [<sup>14</sup>C]Lipid II labeled in the  
604 GlcNAc moiety was prepared as described previously (3). The C<sub>55</sub>-PP-MurNAc(-L-Ala- $\gamma$ -D-  
605 Glu-*meso*-DAP-D-Ala-D-Ala)-GlcNAc lipid II from *E. coli* was used in this study, where  
606 DAP represents diaminopimelic acid. *N*-Dodecyl- $\beta$ -D-maltopyranoside (DDM) was  
607 purchased from Thermo Fisher Scientific, isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG) from  
608 Eurogentec and Ni<sup>2+</sup>-NTA agarose from Qiagen. Colicin M was purified as described  
609 previously (5). Mutanolysin, antibiotics and reagents were from Sigma. Oligonucleotide  
610 synthesis and DNA sequencing were performed by Eurofins-MWG.

611

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617

618 **Conflict of interest:** The authors declare that they have no conflict of interest with the  
619 contents of this article.

620

## 621 FOOTNOTES

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626

627 The abbreviations used are: C<sub>55</sub>-P, undecaprenyl-phosphate; C<sub>55</sub>-PP, undecaprenyl-  
628 pyrophosphate; C<sub>55</sub>-OH, undecaprenol; GGR, geranylgeranyl reductase; Ni<sup>2+</sup>-NTA agarose,  
629 nickel-nitrilotriacetate agarose; DDM, *n*-dodecyl-β-D-maltopyranoside; GlcNAc, *N*-  
630 acetylglucosamine; MurNAc, *N*-acetylmuramic acid; lipid I, undecaprenyl-PP-MurNAc-  
631 pentapeptide; lipid II, undecaprenyl-PP-MurNAc(-pentapeptide)-GlcNAc; DAP,  
632 diaminopimelic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel  
633 electrophoresis; IPTG, isopropyl-β-D-thiogalactopyranoside.

634

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791

792

793 **FIGURE LEGENDS**

794 **Fig. 1: Effect of the overexpression of wild-type and mutant CbrA proteins on *E. coli***  
795 **cells susceptibility to colicin M.** A and C, spot killing assays of the capability of wild-type  
796 and mutated CbrA proteins to protect *E. coli* cells against ColM. In A, spots (5  $\mu$ l) of serial  
797 10-fold dilutions (from top to bottom) of ColM stock (1 mg/ml) were deposited onto lawns of  
798 BW25113 strains (*ca.*  $10^8$  cells) on 2YT-agar plates and growth inhibition zones were  
799 observed after 24 h of incubation at 37°C. In C, 5- $\mu$ l spots of non-diluted (nd, *ca.*  $10^7$  cells) or  
800 serial 10-fold dilutions of cultures of the same strains were deposited onto 2YT-agar plates  
801 containing ColM at the indicated concentrations (0, 10 and 50 ng/ml from left to right,  
802 respectively) and growth was observed after 24 h of incubation at 37°C. CbrA variants  
803 overexpressed by these strains are indicated above the lanes. Control, cells carrying the empty  
804 p*Trc99A* vector; WT(His<sub>6</sub>) and WT, cells expressing CbrA with or without a N-terminal His<sub>6</sub>-  
805 tag (pMLD682 and pMLD391 plasmids, respectively); C46A, Y202A, W204A and K208A,  
806 cells expressing the corresponding CbrA mutants (pMLD682-derived pMLD684, pMLD690,  
807 pMLD688 and pMLD692 plasmids, respectively). In B, BW25113 cells carrying either the  
808 p*Trc99A* empty vector or the *cbrA*-expressing derivative plasmid pMLD391 were grown  
809 exponentially at 37°C in 2YT medium. At the time indicated by the arrow (OD = 0.4), pure  
810 ColM was added at the indicated final concentrations (in ng/ml) and growth was followed at  
811 600 nm. Closed and open symbols correspond to the control and *cbrA*-overexpressing strains,  
812 respectively. Representative data from experiments performed at least in triplicate are shown.

813

814 **Fig. 2: Accumulation of dihydro-C<sub>55</sub>-isoprenoids in membranes of CbrA-, but not Cmi-,**  
815 **overexpressing cells.** FB8 cells carrying either the p*Trc99A* vector (A, B), the *cbrA*-

816 expressing plasmid pMLD391 (C, D), or the *cmi*-expressing plasmid pMLD232 (E) were  
817 grown exponentially at 37°C in 2YT medium. When the OD<sub>600nm</sub> reached 0.7, colicin M was  
818 added (B, D) or not (A, C, E) to the cultures at a concentration of 75 ng/ml. Cultures were  
819 stopped 10 min later and lipids were extracted from the cell pellets by using the Bligh and  
820 Dyer procedure (53). Chloroform extracts were analyzed by reverse-phase HPLC on a  
821 Nucleosil 100 C<sub>18</sub> column (5 μm, 250 × 4.6 mm), using 2-propanol:methanol 1:4 (v/v)  
822 containing 10 mM phosphoric acid as the eluent at a flow rate of 0.6 ml/min. Peaks were  
823 detected at 210 nm. Experiments were carried out 4 times in each condition.

824

825 **Fig. 3: Mass spectrometry analysis of dihydro-C<sub>55</sub>-P extracted from *E. coli* cells.** Lipid  
826 extracts from *cbrA*-overexpressing cells were analyzed by HPLC as described in the text and  
827 the legend to Fig. 2. The peak corresponding to dihydro-C<sub>55</sub>-P was collected and an aliquot  
828 was analyzed by MALDI-TOF mass spectrometry as described in Materials and Methods. A,  
829 authentic commercial dihydro-C<sub>55</sub>-P standard; B, purified lipid extracted from *E. coli* cell  
830 membranes. Peaks at *m/z* 848.10 (A) and 848.00 (B) were observed that were assigned to be  
831 the [M-H]<sup>-</sup> ions.

832

833 **Fig. 4: CbrA and ColM targeted bonds in C<sub>55</sub>-P carrier lipid derivatives.** The structure  
834 and mixed *E,Z* (*trans/cis*) stereochemistry of the C<sub>55</sub>-P carrier lipid is shown. The site of  
835 cleavage of ColM in the peptidoglycan lipid I and II intermediates is indicated, as well as the  
836 α-prenyl unit that is specifically reduced by the CbrA enzyme (the nature of R remaining to be  
837 identified in that case). ColM is known to specifically hydrolyze the lipid I and lipid II  
838 peptidoglycan intermediates and to be inactive on C<sub>55</sub>-P, C<sub>55</sub>-PP, and UDP-MurNAc-  
839 pentapeptide (3).

840

841 **Fig. 5: ColM activity on natural and dihydro forms of peptidoglycan lipid I**  
842 **intermediate.** C<sub>55</sub>-P and dihydro-C<sub>55</sub>-P were extracted and purified from *E. coli* cell  
843 membranes as described in Materials and Methods and Fig. 2. These lipids were subsequently  
844 used for the *in vitro* synthesis of natural (unreduced) (A, B) and dihydro (C, D) forms of  
845 peptidoglycan lipid intermediate I (C<sub>55</sub>-PP-MurNAc-pentapeptide) using purified MraY  
846 enzyme and radiolabeled UDP-MurNAc-[<sup>14</sup>C]pentapeptide (UM5) as co-substrate. These  
847 assays were performed in the absence (A, C) or presence (B, D) of ColM. Radiolabeled  
848 substrate (UM5) and products (lipid I and 1-PP-MurNAc-pentapeptide) were separated by  
849 TLC and detected with a radioactivity scanner, as detailed in the text.

850

851 **Fig. 6: ColM activity on natural and dihydro forms of peptidoglycan lipid II**  
852 **intermediate.** Radiolabeled lipids II were synthesized as described in the text using purified  
853 MraY and MurG enzymes, UDP-MurNAc-pentapeptide, UDP-[<sup>14</sup>C]GlcNAc, and either C<sub>55</sub>-P  
854 or dihydro-C<sub>55</sub>-P as substrates. The natural (unreduced) (A, B) and dihydro (C, D) forms of  
855 [<sup>14</sup>C]lipid II thus generated were purified and incubated in the absence (A, C) or presence (B,  
856 D) of ColM. The radiolabeled substrates (lipids II) and reaction product 1-PP-MurNAc(-  
857 pentapeptide)-GlcNAc were separated by TLC and detected with a radioactivity scanner, as  
858 detailed in the text.

859

860 **Fig. 7: Susceptibility to ColM and resistance conferred by CbrA in transglycosylase and**  
861 **C<sub>55</sub>-PP phosphatase mutants.** BW25113 mutant strains carrying deletions of genes encoding  
862 peptidoglycan transglycosylases or C<sub>55</sub>-PP phosphatases were transformed by the control  
863 vector pUCP24Nco or the *cbrA*-expressing plasmid pMLD679. These strains were grown  
864 overnight in 2YT-gentamicin liquid medium and 5- $\mu$ l spots of non-diluted (nd) or serial 10-  
865 fold dilutions of these cultures were deposited onto 2YT-agar plates containing (B, D) or not

866 (A, C) ColM at a concentration of 15 ng/ml. Growth was observed after 24 h of incubation at  
867 37°C. – and + indicate the absence (empty vector) or presence (pMLD679) of *cbrA*-  
868 overexpressing plasmid, respectively. Numbers 1 to 7 correspond to the following strains: 1,  
869 wild-type BW25113 parental strain; 2,  $\Delta mrcA$  (PBP1A mutant); 3,  $\Delta mrcB$  (PBP1B mutant);  
870 4,  $\Delta mrcB \Delta pbpC \Delta mtgA$  (PBP1B/PBP1C/MtgA triple mutant); 5,  $\Delta pgpB \Delta lpxT \Delta bacA$ ; 6,  
871  $\Delta ybjG \Delta lpxT \Delta bacA$ ; and 7,  $\Delta ybjG \Delta lpxT \Delta pgpB$ . YbjG, PgpB or BacA is the only remaining  
872 C<sub>55</sub>-PP phosphatase expressed in the latter triple mutant strains 5, 6 and 7, respectively.  
873 Representative data from experiments performed at least in triplicate are shown.

874

875 **Fig. 8: Sequence alignment of CbrA and geranylgeranyl reductases.** Homologous  
876 geranylgeranyl reductase protein sequences were retrieved by BlastP. Amino acid sequences  
877 of CbrA from *E. coli* (Ec\_CbrA) and geranylgeranyl reductases from *S. acidocaldarius*  
878 (Sa\_GGR) and *T. acidophilum* (Ta\_GGR), whose structures are solved, plus other GGR  
879 annotated proteins, whose accession numbers are given, were aligned using the constraint-  
880 based multiple protein alignment tool (COBALT) from NCBI (54). Conserved residues are  
881 written in red in sequence blocks. Conserved motifs shared by members of the *para*-  
882 hydroxybenzoate hydroxylase family (PHBH) that are involved in nucleotide and FAD  
883 binding are indicated below the sequences in red and orange, respectively. The motif that is  
884 unique to the GGR sub-family and is involved in lipid binding is indicated in green.  
885 Secondary structure elements based on the 3D structure of Sa\_GGR (PDB accession number:  
886 3ATR) are presented on top:  $\alpha$ -helices ( $\alpha$ ) and  $3_{10}$ -helices ( $\eta$ ) are displayed as squiggles,  $\beta$ -  
887 strands ( $\beta$ ) as arrows, turns with T letters. The figure was generated by using ESPript-3.0  
888 (55). Accession numbers of Sa\_GGR, Ec\_CbrA and Ta\_GGR are WP\_011277849,  
889 WP\_001340434 and WP\_010900941, respectively.

890

891

892

893 **Table 1: Pools of C<sub>55</sub>-isoprenoids in *E. coli* cell membranes**

894 FB8 cells carrying either the empty vector p*Trc99A* or the *cbrA*-expressing plasmid  
 895 pMLD391 were grown exponentially at 37°C in 2YT medium. In all cases, cultures were  
 896 stopped at OD = 1.0 and divided into two 50-ml samples which were treated by the Bligh and  
 897 Dyer (53) and Kato (52) procedures, respectively, as described previously (16). The extracted  
 898 isoprenoids were analyzed by HPLC as described in Materials and Methods and Fig. 2.

899

Strain	IPTG <sup>a</sup>	ColM <sup>a</sup>	Pool level (nmol/g of cell dry weight)						
			C <sub>55</sub> -P	C <sub>55</sub> -PP	C <sub>55</sub> -OH	Dihydro-C <sub>55</sub> -P	Dihydro-C <sub>55</sub> -PP	Dihydro-C <sub>55</sub> -OH	Total
FB8 (p <i>Trc99A</i> )	-	-	246 ± 37	137 ± 25	ND <sup>b</sup>	ND	ND	ND	383 ± 44
FB8 (p <i>Trc99A</i> )	-	+	94 ± 12	52 ± 8	102 ± 19	ND	ND	ND	248 ± 24
FB8 (pMLD391)	-	-	186 ± 26	74 ± 15	ND	61 ± 15	65 ± 18	ND	386 ± 38
FB8 (pMLD391)	+	-	116 ± 23	36 ± 8	ND	113 ± 23	125 ± 24	ND	390 ± 41
FB8 (pMLD391)	-	+	75 ± 15	26 ± 7	47 ± 3	94 ± 19	148 ± 28	ND	390 ± 38
FB8 (pMLD391)	+	+	79 ± 16	5 ± 1	41 ± 6	102 ± 18	164 ± 21	ND	391 ± 32

900

901 Values are expressed as mean ± SD of 4 experiments.

902 <sup>a</sup> + indicates addition of IPTG (0.5 mM) at OD = 0.4 and/or of ColM (75 ng/ml) at OD = 0.7.903 <sup>b</sup> ND, not detected.

904

















