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Involvement of stress protein PspA (phage shock protein A) of *Escherichia coli* in maintenance of the protonmotive force under stress conditions

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The expression of specific PhoE mutant proteins leads to induction of the expression of the *psp* operon of *Escherichia coli* and the export of various plasmid-encoded precursors is retarded in a *pspA* mutant strain. Here, we have investigated the specific role of various Psp proteins in the export process. PspB and PspC are both inner membrane proteins that are involved in the regulation of the transcription of the *psp* operon. Precursor PhoE translocation was retarded in a *pspB* mutant strain to a similar extent as in a *pspA* mutant strain. The reduced translocation efficiencies in the various *psp* mutants could be complemented by expression of PspA from a plasmid, indicating that only PspA is required for efficient translocation. Mutant prePhoE proteins that can be translocated independently of the $\Delta\mu\text{H}^+$ appeared to translocate equally efficiently in a wild-type and in a *pspA* mutant strain. Furthermore, quantitative *in vivo* determination of the $\Delta\mu\text{H}^+$ showed that it specifically decreased in a *pspA* mutant strain upon expression of plasmid-encoded (mutant) prePhoE protein. Apparently, the translocation defects observed in a *psp* mutant strain are caused by a decrease of the $\Delta\mu\text{H}^+$ and PspA functions by maintaining the $\Delta\mu\text{H}^+$ under these conditions.

Keywords: *Escherichia coli*/PhoE protein/protein translocation/protonmotive force/PspA

Introduction

Proteins that have their functional localization in the periplasm or in the outer membrane of *Escherichia coli* are synthesized with an N-terminal extension, the signal sequence, which is essential for translocation across the inner membrane (Milstein *et al.*, 1972). The translocation process involves several proteins, usually designated Sec proteins (Schatz and Beckwith, 1990). SecB is a cytoplasmic protein that interacts with a subset of precursor proteins (Kumamoto, 1989) and targets them to the export machinery in the inner membrane (Hartl *et al.*, 1990; de Cock and Tommassen, 1992). SecA and the integral membrane proteins SecY, SecE and SecG are the constituents of a functional complex (Bieker and Silhavy, 1990; Brundage *et al.*, 1990; Hanada *et al.*, 1994) that

mediates the translocation process, perhaps by forming a gated proteinaceous pore in the membrane (Simon and Blobel, 1992). Finally, SecD and SecF are integral inner membrane proteins that are supposed to function in later steps of the translocation process, e.g. the release of the translocated proteins from the membrane into the periplasm (Gardel *et al.*, 1990; Matsuyama *et al.*, 1993). Precursor protein translocation is an energy-driven process, which requires both ATP hydrolysis and the proton gradient (ΔpH) and electrochemical potential ($\Delta\psi$) components of the protonmotive force (Geller, 1991). *In vitro* studies have shown that SecA, by its translocation-ATPase activity, supplies energy for the initiation of translocation (Lill *et al.*, 1989) and possibly also for later steps in the translocation process (Yamada *et al.*, 1989; Driessen, 1992). The protonmotive force may be the driving force for translocation during periods when SecA protein is not bound to the precursor or at later stages in the translocation process (Geller and Green, 1989; Schiebel *et al.*, 1991). Consistent with the latter suggestion is the reported involvement of SecD and SecF in a protonmotive force-driven step of the translocation reaction (Arkowitz and Wickner, 1994). Alternatively, the protonmotive force might be involved in the opening or widening of the translocation channel, allowing the translocation of (partially) folded structural elements in a precursor (Tani *et al.*, 1990; N.Nouwen, B.de Kruijff and J.Tommassen, manuscript submitted).

In our laboratory, outer membrane protein PhoE is used as a model protein to study protein export in *E.coli*. For efficient translocation across the inner membrane, prePhoE requires the general Sec export pathway. Previously, we have shown that the synthesis of a 26 kDa protein, PspA, is strongly induced upon expression of certain mutant PhoE proteins that are defective in their biogenesis (Kleerebezem and Tommassen, 1993). Also, the overexpression of wild-type *phoE* led, although to a lesser extent, to the induction of the synthesis of PspA and, furthermore, it was demonstrated that the translocation of various precursors is less efficient *in vivo* and *in vitro* in the absence of PspA (Kleerebezem and Tommassen, 1993). The observation that PspA expression is induced in various *sec* mutants (Kleerebezem and Tommassen, 1993) is also consistent with a role of this protein in precursor translocation. PspA (phage shock protein A) was initially identified by Brissette *et al.* (1990) as a stress protein, induced upon infection of *E.coli* cells with filamentous phage f1. PspA expression is also induced under more general stress conditions like heat, ethanol or hyperosmolarity shock (Brissette *et al.*, 1990). The *pspA* gene is the first gene of the stress-induced *psp* operon (Figure 1), which is essential for prolonged stationary phase survival under alkaline conditions (Weiner and Model, 1994). Expression of this operon is modulated by positive and negative feedback

mechanisms (Brissette *et al.*, 1991; Weiner *et al.*, 1991). Based on its amino acid sequence, PspB was suggested to be an integral inner membrane protein, anchored in the inner membrane by a hydrophobic N-terminal segment of 24 amino acid residues (Brissette *et al.*, 1991). It is involved in induction of the expression of the *psp* operon (Weiner *et al.*, 1991). PspC was suggested to act as a positive regulator of the transcription of the *psp* operon. It contains a leucine zipper motif in its C terminus (Brissette *et al.*, 1991; Weiner *et al.*, 1991), a motif that is present in several eukaryotic transcription factors. PspA participates in a negative feedback loop, probably preventing induction of *psp* expression under normal growth conditions (Weiner *et al.*, 1991). A product of the putative fourth open reading frame of the *psp* operon, designated *pspD*, has never been detected *in vivo* or *in vitro*, and *pspE*, encoding a periplasmic protein, is transcribed as part of the operon and from its own promoter (Brissette *et al.*, 1991).

In the present study, we have investigated the role of individual Psp proteins in the translocation process. We demonstrate that both PspB and PspC are inner membrane proteins, involved in the regulation of *psp* expression upon prePhoE overexpression. Furthermore, we show that only

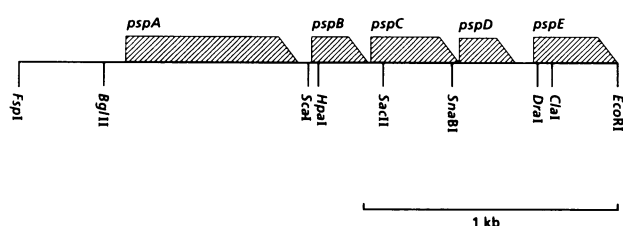


Fig. 1. Schematic representation of the *FspI-EcoRI* DNA fragment obtained from recombinant phage λ IC2. The positions of the *psp* genes and relevant restriction sites are indicated. Insertion of the *kan* resistance cassette into the *HpaI* site resulted in *pspB::kan* (strain CE1417), insertion into the *SacII* site resulted in *pspC::kan* (strain CE1418) and replacement of the *HpaI-SnaBI* fragment by the *kan* cassette led to Δ *pspB-pspC::kan* (strain CE1419).

PspA production is essential for efficient prePhoE translocation and that it supports efficient precursor translocation, probably by maintaining sufficient protonmotive force under stress conditions.

Results

Cloning of the *psp* operon

The *pspA* gene is the first gene of an operon (Figure 1) and, consequently, the *pspA* mutation used in previous experiments (Kleerebezem and Tommassen, 1993) may affect the expression of the downstream located genes. To be able to study the involvement of individual Psp proteins in the translocation process, the *psp* operon was cloned as a 2.35 kb *FspI-EcoRI* fragment (Figure 1) from the recombinant phage λ IC2 (Kohara *et al.*, 1987) in pACYC184, resulting in pJP378. The identity of the cloned fragment was confirmed by comparison of the restriction map of the plasmid with the published nucleotide sequence of the *psp* operon (Brissette *et al.*, 1991). Furthermore, the PspA protein could be detected in whole-cell samples of *pspA* mutant strain CE1343 containing pJP378 by Western blot analysis (results not shown). Subsequently, parts of the *psp* operon were cloned behind the *tac* promoter of expression vector pJF119HE (see Materials and methods and Table I) to investigate the characteristics of the individual Psp proteins.

Subcellular localization of PspB and PspC

PspB was suggested to be an inner membrane protein because of the presence of a hydrophobic N-terminal segment and PspC was suggested to act as a transcriptional activator of the *psp* operon (Brissette *et al.*, 1991; Weiner *et al.*, 1991). However, we noticed a stretch of 26 hydrophobic amino acids (residues 39–64) in the sequence of PspC (Brissette *et al.*, 1991) as well, suggesting that PspC might also be an integral membrane protein. To investigate the subcellular localization of PspB and PspC, expression of the *psp* genes under *tac* promoter control

Table I. Plasmids and their relevant characteristics and sources

Plasmid	Relevant characteristics ^a	Source
pACYC184	Tet ^r , Cam ^r	Chang and Cohen (1978)
pUC4K	Kan ^r , Amp ^r	Vieira and Messing (1982)
pPHO7	Amp ^r , <i>phoA</i> cassette	Gutierrez and Devedjian (1989)
pJF119HE	Amp ^r , <i>tac</i> promoter and <i>lacI^q</i>	Fürste <i>et al.</i> (1986)
pJP29	Cam ^r , wild-type <i>phoE</i> gene	Bosch <i>et al.</i> (1986)
pMR05H2	Cam ^r , mutant <i>phoE</i> gene, amino acids LALVLALV inserted between amino acid residues 158 and 159 of PhoE	Agterberg <i>et al.</i> (1990)
pNN7	Cam ^r , mutant <i>phoE</i> gene, amino acid Gly ⁻¹⁰ substituted by Cys	N.Nouwen
pNN8	Cam ^r , mutant <i>phoE</i> gene, amino acid Gly ⁻¹⁰ substituted by Leu	N.Nouwen
pJP378	Tet ^r , <i>FspI-EcoRI</i> fragment from λ IC2 (containing the <i>psp</i> operon) cloned in pACYC184	This study
pJP379	Amp ^r , <i>pspB</i> behind <i>tac</i> promoter of pJF119HE	This study
pJP380	Amp ^r , <i>pspA-E</i> behind <i>tac</i> promoter of pJF119HE	This study
pJP381	Amp ^r , <i>pspA</i> behind <i>tac</i> promoter of pJF119HE	This study
pJP382	Amp ^r , <i>pspB-E</i> behind <i>tac</i> promoter of pJF119HE	This study
pJP383	Amp ^r , <i>pspB-D</i> behind <i>tac</i> promoter of pJF119HE	This study
pJP384	Tet ^r , Kan ^r , <i>pspB::kan</i> derivative of pJP378	This study
pJP385	Amp ^r , Kan ^r , <i>pspC::kan</i> derivative of pJP380	This study
pJP386	Tet ^r , Kan ^r , Δ <i>pspB-pspC::kan</i> derivative of pJP378	This study
pJP387	Amp ^r , <i>pspC-phoA</i> ; <i>phoA</i> cassette inserted in <i>SacII</i> site of pJP383	This study
pJP388	Amp ^r , <i>pspC-phoA</i> ; <i>phoA</i> cassette inserted in <i>SnaBI</i> site of pJP383	This study
pJP389	Amp ^r , <i>pspE-phoA</i> ; <i>phoA</i> cassette inserted in <i>ClaI</i> site of pJP380	This study

^aTet^r, Cam^r, Amp^r and Kan^r indicate resistance to tetracycline, chloramphenicol, ampicillin and kanamycin, respectively.

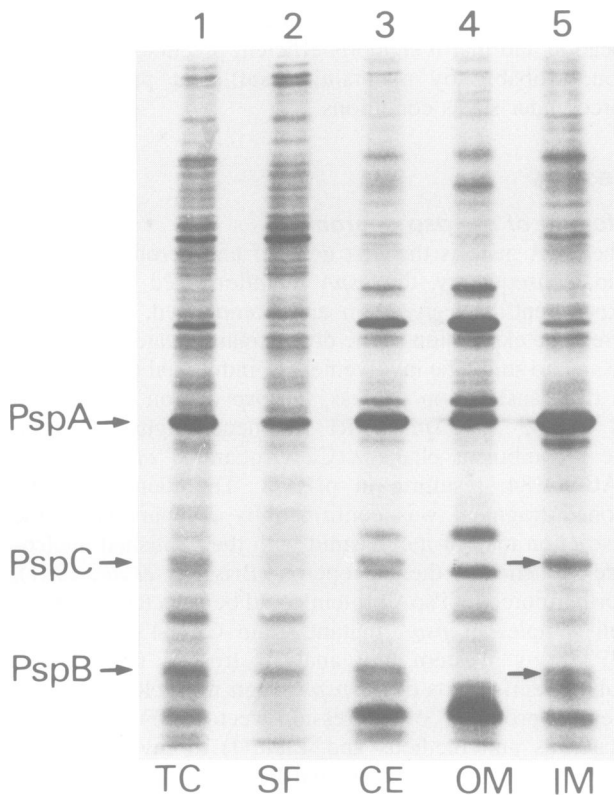


Fig. 2. Subcellular localization of PspB and PspC. Strain CE1224 containing pJP380 (*pspA-pspE*) was grown in HEPES-buffered synthetic medium, supplemented with 660 μM K_2HPO_4 , and expression of the plasmid-encoded *psp* genes was induced by the addition of 45 μM IPTG. After 45 min incubation, cells were pulse labelled for 60 s with [^{35}S]methionine, followed by a 30 min chase. Cells were harvested (TC, lane 1) and samples were subsequently fractionated. The combined periplasmic and cytoplasmic soluble fraction (SF), and the pelleted cell envelope fraction (CE), were obtained after ultrasonic disintegration of the cells. The outer membrane (OM) and inner membrane (IM) fractions were obtained after selective solubilization of the inner membrane in Triton X-100. The fractions obtained were analysed by SDS-PAGE, followed by autoradiography.

was induced by growing strain CE1224 containing pJP380 (*pspA-pspE*) in the presence of 45 μM isopropyl- β -D-thiogalactopyranoside (IPTG). Cells were pulse labelled with [^{35}S]methionine, followed by a chase with an excess of non-radioactive methionine. Subsequently, radiolabelled cells were fractionated (Figure 2). PspA mainly fractionated with the cell envelope (Figure 2, lane 3), but also to some extent with the soluble fraction (lane 2). Upon subfractionation of the cell envelope, PspA mainly co-fractionated with the inner membrane fraction (lane 5). The presence of some PspA in the outer membrane fraction (lane 4) might be due to aggregation of the protein, caused by the high production level. These results are consistent with previously published data (Kleerebezem and Tommassen, 1993). The protein bands representing PspB and PspC were identified by comparing whole-cell protein patterns of pulse-labelled samples of strain CE1224 containing pJF119HE, pJP379, pJP380, pJP382 or pJP383 after IPTG induction (results not shown). Both PspB and PspC appeared in the cell envelope fraction (Figure 2, lane 3) and could be solubilized by Triton X-100 extraction (Figure 2, lane 5), which indicates that both proteins

Table II. Alkaline phosphatase activities measured in cells of strain CE1224 containing pJP387, pJP388 or pJP389 (Table I)

Plasmid	Alkaline phosphatase activity	
	-IPTG	+IPTG
pJP387	0.2	0.4
pJP388	0.5	13.2
pJP389	0.7	15.2

Cells were grown to an OD_{660} of 0.25, and plasmid-encoded hybrid protein synthesis was either not induced (-IPTG) or induced with 100 μM IPTG (+IPTG). Alkaline phosphatase activities are given as nmol *p*-nitrophenol released/min/mg of cells (dry weight).

are inner membrane proteins (Schnaitman, 1974) and is consistent with the presence of hydrophobic stretches in their amino acid sequences.

Application of the positive-inside rule (von Heijne, 1992) to the amino acid sequence of PspC suggests a topology with the N terminus on the cytoplasmic side and the C terminus on the periplasmic side of the inner membrane. To examine whether this topology prediction is correct, pJP387 and pJP388 were constructed (see Materials and methods and Table I), each carrying a *pspC-phoA* gene fusion with the fusion point either before (after codon 19) or after (after codon 112) the putative membrane-spanning segment of PspC, respectively. As a positive control, pJP389 was constructed, carrying a *pspE-phoA* gene fusion with the fusion point C-terminally of the signal sequence of PspE. After induction with IPTG, Psp-PhoA fusion products with the expected apparent molecular weights could be detected by Western blot analysis, using antibodies directed against PhoA (results not shown). However, alkaline phosphatase activity could only be detected in cells containing pJP388 (encoding the larger PspC-PhoA hybrid) or pJP389 (encoding the PspE-PhoA hybrid), but not in cells containing pJP387 (encoding the smaller PspC-PhoA hybrid) (Table II). These results show that the N-terminal 112 amino acid residues of PspC, which include the hydrophobic segment, can mediate the export of alkaline phosphatase, thus confirming the inner membrane localization and the predicted topology of PspC. Hence, both PspB and PspC are integral inner membrane proteins, and the leucine zipper motif within the C-terminal end of PspC is translocated to the periplasmic side of the membrane.

PspB- and PspC-mediated regulation of expression of the *pspA* gene

PspB and PspC have been shown to be involved in the regulation of expression of the *psp* operon at the transcriptional level (Brissette *et al.*, 1991; Weiner *et al.*, 1991). To assess whether PspB and PspC are also required for the induction of PspA synthesis upon overexpression of (mutant forms of) prePhoE protein, chromosomal knock-out mutations in the *pspB* and *pspC* genes were introduced in strain CE1224 (see Materials and methods and Table III). The resulting *psp* mutant strains were transformed with either pJP29, encoding wild-type prePhoE, or pMR05H2, encoding a mutant form of prePhoE (Table I), the expression of which results in strong induction of PspA synthesis (Kleerebezem and Tommassen, 1993). As expected, when wild-type strain

Table III. Bacterial strains and their characteristics and sources

Strain	Properties	Source ^a /reference
AM1095	F ⁻ , <i>leu thi trp his pdxA pyrA lacY galK xyl ara mtl fhuA tsx rpsL recB21 recC22 sbcB15 sup</i>	Hoekstra <i>et al.</i> (1980)
GM48	F ⁻ , <i>thr leu thi dam3 dcm6 lacY galK galT mtl fhuA tsx supE</i>	PC2953
CE1224	F ⁻ , <i>thr leu Δ(<i>proA-proB-phoE-gpt</i>) his thi argE lacY galK xyl rpsL supE ompR</i>	Tommassen <i>et al.</i> (1983)
CE1343	<i>pspA::kan</i> derivative of CE1224	Kleerebezem and Tommassen (1993)
CE1417	<i>pspB::kan</i> derivative of CE1224	This study
CE1418	<i>pspC::kan</i> derivative of CE1224	This study
CE1419	Δ <i>pspB-pspC::kan</i> derivative of CE1224	This study

^aPC strain was obtained from the N.C.C. Phabagen collection, Utrecht University, Padualaan 8, The Netherlands (F.van Asma, curator).

CE1224 containing pJP29 was grown under phosphate limitation to induce PhoE synthesis, induction of PspA protein production was observed, and this induction was even stronger in the case of the cells containing pMR05H2 (Figure 3). In both the *pspB::kan* strain CE1417 and the Δ *pspB-pspC::kan* strain CE1419, the physiological level of PspA production was already slightly reduced as compared with that in the wild-type strain CE1224 (Figure 3, lanes 1), and induction of PspA overproduction upon (mutant) PhoE expression appeared to be strongly reduced, but not completely abolished (Figure 3, lanes 2 and 3). Possibly, the *pspB::kan* mutation in strain CE1417 has a polar effect on the expression of the *pspC* gene, resulting in a *pspB pspC* phenotype in this strain. However, in the *pspC::kan* strain CE1418, only a slight reduction of PspA overproduction relative to the parental strain CE1224 could be detected (Figure 3), indicating that PspC plays only a minor role in the regulation of PspA expression under these conditions. The effects on the expression level of PspA observed in the *pspB* and/or *pspC* mutant strains could be completely complemented by expression of the corresponding genes *in trans* (from plasmid pJP383, containing *pspB-D* under *tac* promoter control; results not shown). Remarkably, in all *psp* mutant strains tested, but in contrast to the wild-type strain, the level of PspA synthesis was similar in cells containing pJP29 or pMR05H2 (Figure 3, compare lanes 2 and 3). Taken together, although *psp* induction upon expression of (mutant) prePhoE is not completely abolished in the *psp* mutants, both PspB and PspC seem to be required for PspA overproduction, and PspB appears to play a more important role in this respect than PspC.

Processing kinetics of prePhoE in *psp* mutants

The translocation of prePhoE and other precursors was shown to be retarded when they were overproduced in a *pspA::kan* mutant (Kleerebezem and Tommassen, 1993). Since the *pspA::kan* mutation used could have a polar effect on the expression of the genes located downstream in the operon, the results of those experiments do not allow it to be concluded whether PspA protein itself or the product of any of the other *psp* genes is required for efficient translocation. Therefore, we investigated whether the newly constructed *pspB* and *pspC* mutations also influenced the prePhoE translocation kinetics. The wild-type strain CE1224 and the *psp* mutant strains containing pJP29 were pulse labelled, and processing of prePhoE was followed during the chase (Figure 4). Translocation of wild-type prePhoE, as monitored by the processing kinetics, was retarded in both the *pspB* mutant strain

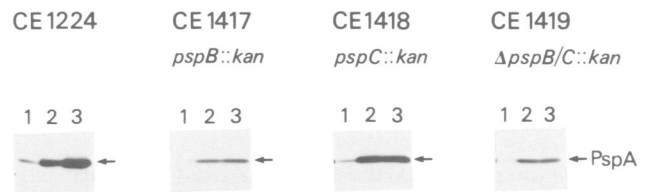


Fig. 3. Induction of PspA expression in *psp::kan* mutant strains. Cells of strain CE1224 (wild type), CE1417 (*pspB::kan*), CE1418 (*pspC::kan*) and CE1419 (Δ *pspB-C::kan*) containing either no plasmid (lanes 1), or plasmids pJP29 (lanes 2) or pMR05H2 (lanes 3) were grown under phosphate limitation. Equal amounts of cells of the various strains (determined by OD measurement of the cultures) were loaded onto the gels and analysed by Western blotting, using anti-PspA antiserum.

CE1417 and in the Δ *pspB-pspC* mutant strain CE1419 to a similar extent as in the *pspA* mutant strain CE1343. However, in the *pspC* mutant strain CE1418, prePhoE was apparently translocated almost as efficiently as in the wild-type strain CE1224 (Figure 4). These results indicate either that the reduced amounts of PspA produced in the *pspB* and Δ *pspB-pspC* mutants upon expression of prePhoE (Figure 3) result in reduced translocation kinetics of prePhoE, or that especially PspB, possibly in addition to PspA, plays a role in precursor protein translocation.

Complementation of translocation defects in *psp::kan* strains by expression of plasmid-encoded *psp* genes

Since it remained unclear which of the *psp* gene products is actually involved in maintaining the efficient kinetics of prePhoE translocation, complementation experiments were performed. Strains CE1343 (*pspA::kan*) and CE1419 (Δ *pspB-pspC::kan*), both containing pJP29, were transformed with pJP380, pJP381 or pJP382, each containing a part of the *psp* operon under *tac* promoter control (Table I). The cells were grown under phosphate-limiting conditions to induce the expression of prePhoE, and the expression of the plasmid-encoded *psp* genes was induced by addition of 25 μM IPTG to the medium. The cells were pulse labelled and chased, and PhoE protein was immunoprecipitated from the samples. The prePhoE translocation defect observed in both the *pspA* mutant strains CE1343 (Figure 5B) and in the Δ *pspBC* mutant strain CE1419 (Figure 5C) could be completely complemented to wild-type level (Figure 5A) when either all Psp proteins (pJP380) or only PspA (pJP381) were expressed *in trans*. On the other hand, expression of PspB–PspE from plasmid pJP382 could only complement the translocation defect

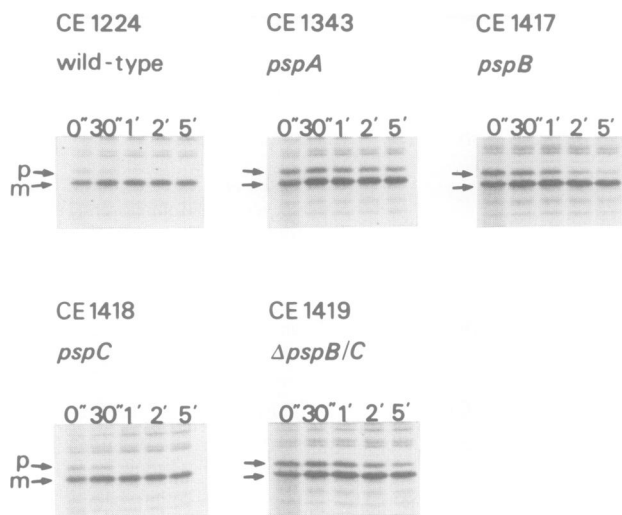


Fig. 4. *In vivo* processing kinetics of pJP29-encoded prePhoE in strains CE1224 (wild-type) and various *psp* mutant derivatives. Cells were starved for phosphate and pulse labelled for 30 s with [³⁵S]methionine, followed by chase periods as indicated. Protein patterns were analysed by SDS-PAGE, followed by autoradiography. Only the relevant part of the autoradiograms is shown. Indicated are precursor (p) and mature (m) PhoE bands.

in the Δ *pspBC* mutant CE1419, but not in the *pspA* mutant CE1343 (Figure 5). Since expression of only PspA from a plasmid is sufficient to overcome the translocation defect in the Δ *pspBC* strain CE1419, it must be concluded that the reduced translocation kinetics in this strain are caused by the reduced induction level of PspA protein upon expression of pJP29-encoded prePhoE. This result indicates that only the high production of PspA is required for efficient protein translocation, and that PspB and PspC are merely required for the regulation of PspA production.

The role of PspA in translocation

Whereas it is clear from the results presented that PspA expression is important for efficient protein translocation, its exact role in this process remains to be determined. The *psp* operon has been shown to be essential for prolonged stationary phase survival under alkaline conditions, and PspA production has been shown to be induced by addition of the proton ionophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) to the medium (Weiner and Model, 1994). Both conditions can be interpreted as stress conditions that undermine the $\Delta\mu\text{H}^+$ of the cell. Thus, dissipation of the $\Delta\mu\text{H}^+$, which could also occur when the export apparatus is overloaded by overexpression of *phoE* from a plasmid, could be the trigger for *psp* induction, and PspA could possibly play a role in maintaining the $\Delta\mu\text{H}^+$ under these conditions. The observed export defects in a *pspA* mutant could then be a consequence of the loss of $\Delta\mu\text{H}^+$, which is essential for efficient prePhoE translocation (de Vrije *et al.*, 1987). If this hypothesis is correct, one would expect that translocation of a precursor which is less dependent on the $\Delta\mu\text{H}^+$ for its translocation is unaffected by the *pspA* mutation. To study this possibility, the translocation kinetics of two mutant prePhoE proteins were studied in the *pspA* mutant strain CE1343. The mutant prePhoE proteins encoded by pNN7 and pNN8 each contain a single amino acid

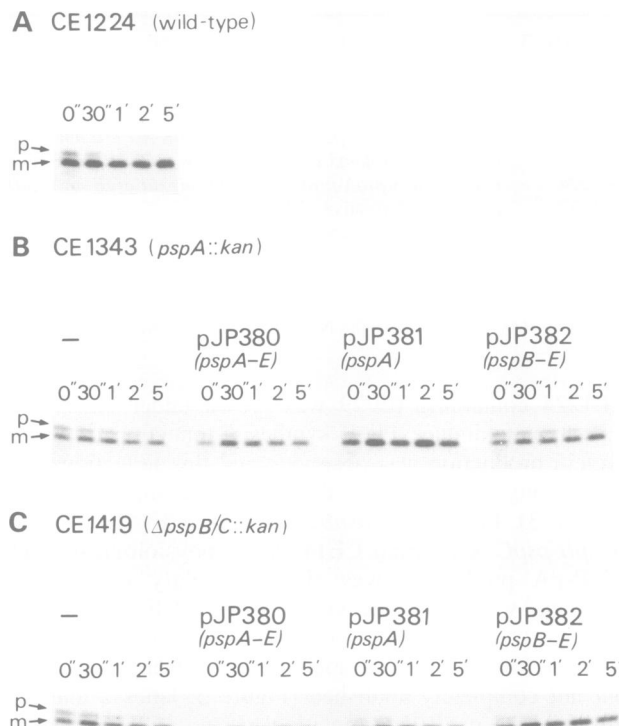


Fig. 5. *In vivo* processing kinetics of pJP29-encoded prePhoE protein in strains CE1224 (A) and its *psp* mutant derivatives CE1343 (B) and CE1419 (C), containing either no additional plasmid (lanes -) or plasmids pJP380 (containing *pspA-pspE*), pJP381 (containing *pspA*) or pJP382 (containing *pspB-pspE*). The cells were starved for phosphate and pulse labelled for 30 s with [³⁵S]methionine, followed by chase periods as indicated. PhoE was immunoprecipitated from the samples and analysed by SDS-PAGE, followed by autoradiography. Indicated are the precursor (p) and mature (m) PhoE bands.

substitution at position -10 (Gly⁻¹⁰ to Cys and Gly⁻¹⁰ to Leu, respectively) in the signal sequence, resulting in a relief of the $\Delta\mu\text{H}^+$ dependency of translocation (Nouwen *et al.*, 1996). In pulse-chase experiments, it appeared that the pNN7- and pNN8-encoded prePhoE proteins, in contrast to the wild-type prePhoE (Figure 4), could be translocated as efficiently in the *pspA* mutant strain CE1343 as in the parental strain CE1224 (Figure 6). These results support the hypothesis that the reduced export efficiency of wild-type prePhoE in a *pspA* mutant strain is a consequence of a decrease in $\Delta\mu\text{H}^+$ when the export machinery is overloaded, and suggest that PspA is required to maintain the $\Delta\mu\text{H}^+$ under these stress conditions.

PspA functions in maintaining the $\Delta\mu\text{H}^+$

If PspA plays a role in maintenance of the $\Delta\mu\text{H}^+$ under stress conditions, one would expect a more rapid loss of the $\Delta\mu\text{H}^+$ under PspA-inducing stress conditions in a *pspA* mutant strain, when compared with a wild-type strain. To investigate this possibility, $\Delta\psi$ was measured *in vivo* using a tetraphenylphosphonium (TPP⁺)-selective electrode (see Materials and methods); these experiments have been performed repeatedly with essentially the same results. Control experiments in which nigericin was added during the measurement, resulting in conversion of the ΔpH component of the $\Delta\mu\text{H}^+$ into a $\Delta\psi$ and thereby allowing the measurement of the total $\Delta\mu\text{H}^+$ by measure-

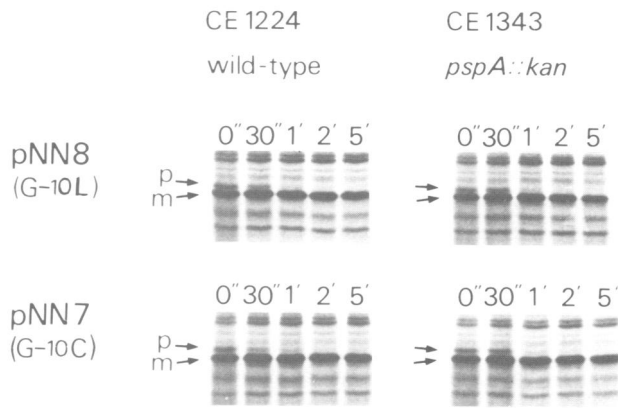


Fig. 6. *In vivo* processing of pNN8- and pNN7-encoded prePhoE proteins in strain CE1224 (left panels) and in its *pspA* mutant derivative CE1343 (right panels). The cells were starved for phosphate and pulse labelled for 30 s with [³⁵S]methionine, followed by chase periods as indicated. Protein patterns were analysed by SDS-PAGE, followed by autoradiography. Only the relevant part of the autoradiograms is shown. Indicated are precursor (p) and mature (m) PhoE bands.

ment of only the $\Delta\psi$, showed a relatively small effect of the addition of nigericin (<10 mV, results not shown), indicating that when grown under these conditions the cells maintain a $\Delta\mu H^+$ that consists mainly of a $\Delta\psi$. The $\Delta\mu H^+$ was determined in EDTA-treated cells of pJP29-containing derivatives of strain CE1224 and *pspA* mutant CE1343, prior to, and 60 or 105 min after induction of prePhoE synthesis. In the uninduced cells (expression of the *phoE* gene has not been induced), the membrane potential was approximately the same in both strains (Figure 7) and had a magnitude that is comparable with previous measurements where values had been corrected for concentration-dependent probe binding to cellular components (Elferink *et al.*, 1985), indicating that the *pspA* mutation in strain CE1343 does not affect the magnitude of the $\Delta\mu H^+$ under normal conditions, i.e. when no plasmid-encoded prePhoE expression is induced. In the wild-type strain, the $\Delta\mu H^+$ was only slightly reduced upon prolonged prePhoE expression (Figure 7, open circles), whereas it decreased in the *pspA* mutant (Figure 7, closed circles). Expression of pMR05H2-encoded mutant PhoE protein induces the expression of PspA in CE1224 much more strongly than the pJP29-encoded wild-type prePhoE does (Kleerebezem and Tommassen, 1993). Therefore, the effect of the expression of this mutant protein on the $\Delta\mu H^+$ was also determined. In the wild-type strain, only prolonged induction of the expression of the pMR05H2-encoded mutant prePhoE resulted in a slight reduction of the $\Delta\mu H^+$ (Figure 7, open squares). However, in the *pspA* mutant strain CE1343, the $\Delta\mu H^+$ decreased dramatically upon expression of this mutant prePhoE (Figure 7, filled squares). These results show that PspA indeed plays a role in the maintenance of the membrane potential ($\Delta\mu H^+$) when (mutant) prePhoE is overproduced from a plasmid, whereas under normal conditions, the $\Delta\mu H^+$ does not appear to be influenced by the *pspA* mutation.

Discussion

Previously, we have observed that the expression of certain mutant PhoE proteins with a defect in their biogenesis

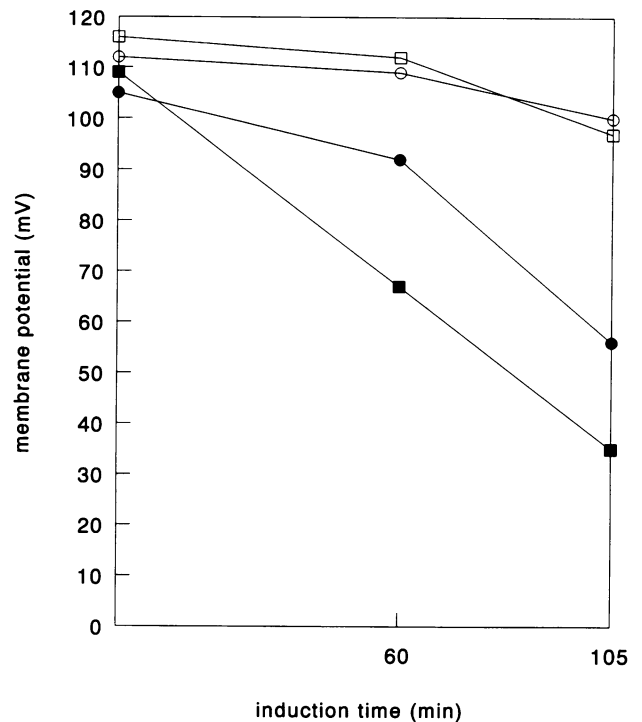


Fig. 7. Quantitative determination of the $\Delta\mu H^+$ *in vivo* in permeabilized cells of pJP29- or pMR05H2-containing derivatives of the strains CE1224 (○ and □, respectively) and *pspA* mutant CE1343 (● and ■, respectively). Cells were grown in HEPES-buffered synthetic medium, supplemented with 660 μM K_2HPO_4 , to mid-log phase and membrane potential measurements were performed using a TPP^+ -selective electrode, either without or after induction of prePhoE synthesis as described in Materials and methods. The membrane potentials given are corrected for TPP^+ binding to cellular components.

and, to a lesser extent, the overproduction of wild-type PhoE leads to the induction of the phage shock response (Kleerebezem and Tommassen, 1993). We have also demonstrated that the export of various precursors, when overproduced from a plasmid, is hampered in a *psp* mutant. Furthermore, it has been demonstrated that dissipation of the $\Delta\mu H^+$ by the uncoupler CCCP leads to the induction of the phage shock response (Weiner and Model, 1994). The results described in the present paper show that there is a correlation between these observations and provide an explanation for the role of the Psp proteins in protein export. It appears that the overproduction of (mutant) prePhoE leads to dissipation of the $\Delta\mu H^+$, which is required for efficient protein export, and that the Psp proteins help to maintain the $\Delta\mu H^+$ under these detrimental conditions. Consistently, the efficiency of the export of mutant prePhoE proteins that are less dependent on the $\Delta\mu H^+$ for their translocation was not affected in a *psp* mutant, in contrast with the export of wild-type prePhoE.

The dissipation of the $\Delta\mu H^+$, observed upon the overproduction of (mutant) prePhoE, could possibly be explained by the accumulation of precursor proteins in the export apparatus in the inner membrane, which could lead to ion leakage through the open export channel (Schiebel and Wickner, 1992; Simon and Blobel, 1992; Kawasaki *et al.*, 1993). This explanation implicates that targeting of the prePhoE protein to the export pathway is essential for the *psp* response. Consistently, the *psp*

response is neither induced upon expression of a prePhoE mutant protein that lacks the signal sequence and that will therefore not be targeted to the export machinery, nor upon overproduction of PhoE in a *secB* null mutant in which proper targeting to the export machinery is disturbed (de Cock and Tommassen, 1992; Kleerebezem and Tommassen, 1993). Furthermore, among the strongest *psp* response-inducing mutant prePhoE proteins are those for which the release from the inner membrane is potentially disturbed (Kleerebezem and Tommassen, 1993), like a mutant protein containing an altered signal peptidase cleavage site (encoded by pJP369) that results in poor processing by leader peptidase or a mutant protein containing a stretch of eight hydrophobic amino acids in its mature domain (encoded by pMR05H2, Table II) that could possibly result in faltering of the translocation process. Similarly, a preLamB mutant protein that contains a mutated signal peptidase cleavage site (LamBA23D), of which the effect on precursor processing is comparable with that observed for the pJP369-encoded mutant prePhoE protein, has been shown to induce the *psp* response to a high level (Carlson and Silhavy, 1993).

Previously, we have shown by Western blot analysis that the production of MalE-LacZ and LamB-LacZ hybrid proteins leads to overproduction of PspA (Kleerebezem and Tommassen, 1993). These hybrid proteins enter the export pathway, where they get stuck (Tommassen and de Kroon, 1987), resulting in the accumulation of other exported proteins (Bassford *et al.*, 1979; Ito *et al.*, 1981). These findings are consistent with the postulated *psp*-inducing signal being dissipation of the $\Delta\mu\text{H}^+$ upon entrance of a precursor protein into and the subsequent blockage of the export apparatus in the inner membrane. Interestingly, we observed that conditional mutations in *secD*, *secF* or *secA*, in contrast to those in *secY* and *secE*, induce PspA synthesis when cells were grown at their restrictive temperature (Kleerebezem and Tommassen, 1993). SecD has been shown to function in the release of translocated precursor proteins from the cytoplasmic membrane (Matsuyama *et al.*, 1993) and, furthermore, it has been shown that depletion of cells for SecD and SecF results in a decrease of the magnitude of the $\Delta\mu\text{H}^+$ (Arkowitz and Wickner, 1994) which, we postulate, is the signal for the *psp* response. Furthermore, the introduction of a *pspA::kan* mutation into the chromosome of *secD1* strain C61 enhanced the cold-sensitive phenotype of the *secD* mutant, resulting in strongly reduced growth of the double mutant at the intermediate temperature of 30°C (M.Kleerebezem, unpublished observation). Recent observations (Chun and Randall, 1994) indicate that the SecA protein in the *secA51* conditional mutant is defective at a stage after it reaches the membrane, suggesting that precursor proteins in this mutant are properly targeted and might accumulate within the translocation apparatus in the inner membrane, resulting in dissipation of the $\Delta\mu\text{H}^+$. In contrast, in the conditional *secY* and *secE* mutants grown at the restrictive temperature, as well as in the *secB* null mutant, the entrance of precursors into the membrane is probably blocked, explaining the absence of the *psp* response in these mutants.

As the *psp* operon contains five genes (Brissette *et al.*, 1991), the question was raised which of the Psp proteins is required for maintaining sufficient $\Delta\mu\text{H}^+$ to support

efficient precursor protein translocation. The *pspD* gene product could not be detected *in vivo* or *in vitro* (Brissette *et al.*, 1991; M.Kleerebezem, unpublished observation), which makes it doubtful whether the *pspD* open reading frame actually constitutes a functional gene. The *pspE* gene is transcribed as part of the operon, but also from its own promoter. Furthermore, deletion of the *pspE* gene has no apparent effect on the *psp* response under inducing conditions (Brissette *et al.*, 1991). Therefore, we decided to concentrate on *pspA*, *pspB* and *pspC*. Based on sequence data, PspB was predicted to be an inner membrane protein (Brissette *et al.*, 1991), which is confirmed by our results. PspC was suggested to function as the cytoplasmic transcriptional activator of the *psp* operon, but it also appears to be an integral inner membrane protein. The leucine zipper motif, which is found in several eukaryotic transcriptional activators, appears to be located at the periplasmic side of the membrane. Thus, the structural organization of PspC is very similar to that of the FtsL protein of *E.coli*, which is involved in cell division. FtsL has a similar mol. wt to PspC (13.6 kDa), and consists of an N-terminal cytoplasmic domain, a single membrane-spanning domain and a C-terminal domain containing a leucine zipper motif in the periplasm (Guzman *et al.*, 1992). Based on their localization in the inner membrane, one could expect PspB and C to play a role in maintenance of the $\Delta\mu\text{H}^+$. However, our complementation experiments indicate that only PspA is required to prevent the loss of $\Delta\mu\text{H}^+$ upon overproduction of prePhoE, whereas PspB and PspC seem to be merely required for the induction of PspA synthesis to a level sufficient to fulfil its function. A tempting model for the regulation of *psp* expression by PspB and C is that PspC could be the sensor for the $\Delta\mu\text{H}^+$ and PspB, although it is anchored to the membrane, could be the transcriptional activator. Such membrane-anchored transcriptional activators have been described. For example, the ToxR protein of *Vibrio cholerae* is an integral inner membrane protein, which plays a role in regulation of expression of the virulence genes of the bacterium and it was shown to bind to the promoter region of the *toxT* gene (Higgins and DiRita, 1994). Alternatively, regulation of transcription of the *psp* operon is mediated by PspB and C via a third regulatory component that has not been identified yet.

Remarkable is the relative importance of PspB and PspC in the regulation of transcription of the *psp* operon under different *psp* inducing conditions. We have found that mainly PspB is required for the induction of the *psp* response upon (mutant) prePhoE expression, whereas PspC only slightly enhances the *psp* response under these conditions. In contrast, it has been reported that PspC is primarily required for the *psp* response to ethanol and hyperosmolarity shock, and that PspB merely enhances the PspC-directed expression under these conditions (Weiner *et al.*, 1991). Upon f1 infection, both PspB and PspC seem to be required for the *psp* response (Weiner *et al.*, 1991). However, neither PspB nor PspC is required for the full *psp* response upon heat shock (Weiner *et al.*, 1991). These variable requirements for PspB and PspC in *psp* induction are intriguing. By measuring the $\Delta\mu\text{H}^+$ *in vivo* under different inducing conditions, it can be established whether the loss of $\Delta\mu\text{H}^+$ is the inducing signal for the *psp* response in all cases.

Whereas it is clear that the PspA protein is responsible for maintaining the $\Delta\mu\text{H}^+$ under the detrimental conditions described and thereby supports efficient precursor protein translocation, an important remaining question is how this protein exerts its function. PspA could be directly involved in precursor protein translocation, possibly by stimulation of one of the components of the translocation machinery, thereby increasing the kinetics of translocation, which could prevent the accumulation of precursor proteins and the loss of $\Delta\mu\text{H}^+$. Alternatively, PspA could be involved in the export process in an indirect manner, e.g. by increasing the activity of the $\Delta\mu\text{H}^+$ -generating systems of the cell, thereby counteracting the loss of $\Delta\mu\text{H}^+$ caused by the applied stress condition. Elucidation of the mechanism by which PspA fulfils its role will be the primary goal for further studies.

Materials and methods

Bacterial strains and growth conditions

The sources and relevant characteristics of the *E. coli* K-12 strains used are listed in Table III. The *pspB::kan*, *pspC::kan* and Δ *pspB-pspC::kan mutations were first constructed on plasmids (pJP384, pJP385 and pJP386, respectively; for a description, see below). These mutations were introduced in the chromosome of *recBC sbcB* strain AM1095 by homologous recombination after transformation with linear plasmid DNA fragments, selecting for kanamycin-resistant transformants. Subsequently, the chromosomal *psp::kan* mutations were introduced in strain CE1224 by P1 transduction (Miller, 1972), resulting in strains CE1417, CE1418 and CE1419 (Table III).*

Unless otherwise notified, bacteria were grown at 30°C in L-broth (Tommasen *et al.*, 1983) or in a synthetic medium in which the phosphate concentration can be varied (Tommasen and Lugtenberg, 1980). When necessary, chloramphenicol (25 µg/ml), kanamycin (100 µg/ml) or ampicillin (50 µg/ml) was added to the media. Conditions for the induction of PhoE protein expression were as described previously (Bosch *et al.*, 1986).

DNA manipulations and plasmids

Plasmid DNA was isolated as described previously (Birnboim and Doly, 1979), followed by anion-exchange chromatography on Qiagen columns (Diagen, Düsseldorf, Germany). DNA from the recombinant phage λ IC2 (Kohara *et al.*, 1987) was isolated according to Sambrook *et al.* (1989) with strain CE1224 as the host for phage replication. Recombinant DNA techniques were performed essentially as described previously (Sambrook *et al.*, 1989). Restriction endonuclease, Klenow fragment of *E. coli* DNA polymerase, T4 DNA polymerase and T4 DNA ligase were used according to the manufacturers' protocols (Pharmacia and New England Biolabs).

Cloning procedures

The plasmids used in this study are listed in Table I. The 2.35 kb *FspI*-*EcoRI* fragment (Figure 1) from the recombinant phage λ IC2 (Kohara *et al.*, 1987) which, based on its chromosomal localization and restriction pattern (Brissette *et al.*, 1990, 1991), was expected to contain the *psp* operon, was cloned in *ScaI*-*EcoRI*-digested pACYC184. The recombinant plasmid was designated pJP378. Several fragments of pJP378, containing the different genes of the *psp* operon, were subcloned behind the *tac* promoter of the pJF119HE expression vector. pJP380, pJP381, pJP382, pJP383 and pJP379 resulted from subcloning the 2 kb *BglII*-*EcoRI* (containing the entire *psp* operon; Figure 1), the 0.8 kb *BglII*-*ScaI* (containing *pspA*), the 1.2 kb *ScaI*-*EcoRI* fragment (containing *pspB-pspE*), the 0.9 kb *ScaI*-*DraI* (containing *pspB-pspD*) and the 0.25 kb *ScaI*-*SacII* (containing *pspB*; the *SacII* site was blunted with T4 DNA polymerase) fragments from pJP378 into pJF119HE, digested with *BamHI*-*EcoRI*, *BamHI*-*SmaI*, *SmaI*-*EcoRI*, *SmaI* and *SmaI*, respectively. To construct the *psp::kan* plasmids pJP384, pJP385 and pJP386, the kanamycin resistance gene (*kan*) cassette, obtained as a 1.3 kb *HincII* fragment from pUC4K, was used. Plasmids pJP384 (containing *pspB::kan*) and pJP385 (containing *pspC::kan*) were constructed by cloning the *kan* cassette in the *HpaI* site of pJP378 and in the *SacII* site (blunted with T4 DNA polymerase) of pJP380, respectively. Deletion

plasmid pJP386, containing the Δ *pspB-pspC::kan* mutation, was constructed by replacing the 0.55 kb *HpaI*-*SnaBI* fragment (Figure 1) of pJP378 by the *kan* cassette.

To construct the in-frame *psp-phaA* gene fusion on plasmids pJP387, pJP388 and pJP389, the *phaA* cassette, obtained as a 2.6 kb *SmaI* fragment from pPHO7, was used. This cassette contains the fragment of the *phaA* gene that encodes the mature part of alkaline phosphatase. The *phaA* cassette was ligated in-frame in the *pspC* gene of pJP383 at the *SacII* (blunted with T4 DNA polymerase) and at the *SnaBI* site (Figure 1), resulting in pJP387 and pJP388, respectively. The *phaA* cassette was also cloned in-frame in the *Clal* site within the *pspE* gene (Figure 1) on pJP382, resulting in pJP389. To obtain non-methylated pJP382, which was necessary to cleave the plasmid with *Clal*, the plasmid was isolated from *dam dcm* mutant strain GM48.

Alkaline phosphatase activity assay

Cells were grown to an OD₆₆₀ of 0.25 in synthetic medium (Tommasen and Lugtenberg, 1980) supplemented with 660 µM K₂HPO₄. Subsequently, growth was continued for 2 h, either in the presence or in the absence of 100 µM IPTG. Alkaline phosphatase activities were determined using *p*-nitrophenyl phosphate as a substrate (Tommasen and Lugtenberg, 1980). Alkaline phosphatase units were calculated as nanomoles of *p*-nitrophenol released per minute per milligram of cells (dry weight).

Isolation and analysis of cell fractions

Cells were grown to an OD₆₆₀ of 0.4 in synthetic medium supplemented with 660 µM K₂HPO₄. After addition of IPTG to a final concentration of 45 µM, growth was continued for 45 min, after which cells were pulse labelled for 60 s with 2.5 µCi [³⁵S]methionine/250 µl of culture and subsequently chased with an excess of non-radioactive methionine for 30 min. Radiolabelled cells were harvested by centrifugation and fractionated. Cell envelopes were isolated by centrifugation after ultrasonic disintegration of cells (Lugtenberg *et al.*, 1975). The supernatant fraction containing the cytoplasmic and periplasmic proteins was concentrated by precipitation with trichloroacetic acid (TCA) (Ito *et al.*, 1981). Inner membranes were separated from outer membranes by selective solubilization in Triton X-100 (Schnaitman, 1974), followed by sedimentation of the outer membrane by centrifugation. Solubilized inner membrane proteins were precipitated from the supernatant with methanol:chloroform (2.4:1). Protein patterns of cell fractions were analysed by SDS-PAGE (Lugtenberg *et al.*, 1975) on gels containing 15% acrylamide, followed by autoradiography.

Western immunoblot analysis

Cells were harvested by centrifugation and solubilized in sample buffer for 10 min at 100°C. Total cellular proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose filters (Schleicher and Schuell, 0.45 µm) using a semi-dry electroblotting apparatus (2117 Multiphor II, LKB). Immunoincubations were performed essentially as described previously (Agterberg *et al.*, 1988), using polyclonal antibodies directed against PspA or PhoA.

Measurement of processing kinetics

Samples (200 µl) were drawn from a culture of phosphate limitation-induced cells that were pulse labelled for 30 s at 30°C with [³⁵S]methionine and chased for varying time periods with an excess of non-radioactive methionine as described previously (Bosch *et al.*, 1986). After precipitation with TCA, radiolabelled proteins were either directly, or after immunoprecipitation (Bosch *et al.*, 1989), separated by SDS-PAGE and analysed by autoradiography.

Quantitative determination of the membrane potential *in vivo*

The membrane potential was quantitatively determined *in vivo* in EDTA-treated cells (Elferink *et al.*, 1985) using the lipophilic cation TPP⁺ assay and correcting the calculated membrane potentials for concentration-dependent probe binding to cellular components (Loikema *et al.*, 1982). Briefly, the electrochemical potential across the cytoplasmic membrane of bacteria can be calculated from the distribution of the lipophilic cation TPP⁺ between the bulk phases of the medium and the cytoplasm. The bulk phase concentration can only be determined when knowledge is available about the distribution of the binding of the probe over the different cellular components. The method used applies models for concentration-dependent probe binding which are based on the assumption that the binding is an energy-independent process. These models allow a proper calculation of the electrochemical potential when the

binding of the probe to the different cellular components is known (Lolkema *et al.*, 1982). The formula used to calculate the membrane potential is given below where C_o = probe concentration in the medium, before adding the cells, C_e = extracellular probe concentration as recorded by the electrode, x = fractional internal volume, $f_{cm(om)}$ = ratio of fractional cytoplasmic membrane (outer membrane) and intracellular volume, $K_{cm(om)}$ = cytoplasmic membrane (outer membrane) partition coefficient and K_i = binding constant for binding to intracellular components.

$$\Delta\psi = Z \log \left\{ \frac{C_o/C_e - 1 + x(1 - 1/2f_{cm}K_{cm} - f_{om}K_{om})}{x(1 + K_i + 1/2f_{cm}K_{cm})} \right\}$$

Cells were grown in HEPES-buffered synthetic medium, supplemented with 660 μ M K_2HPO_4 (HHP) to mid-log phase ($OD_{660} = 0.5$). Subsequently, the cultures were chilled on ice, cells were harvested by centrifugation, washed with HEPES-buffered synthetic medium with no phosphate added (HNP), and cell pellets were resuspended in HNP at a protein concentration of ~ 10 mg/ml. Expression of plasmid-encoded prePhoE was induced by 1:20 dilution of the concentrated cells in HNP and incubation of the cell suspension at 37°C for 60 or 105 min, after which cells were re-isolated and resuspended in HNP at a protein concentration of ~ 10 mg/ml. Shortly before the determination of the membrane potential, cells were resedimented by centrifugation and resuspended in a double volume of 0.9% NaCl, 25 mM HEPES (pH 7.5). Subsequently, 5 mM Na_2EDTA was added, and cells were incubated for 10 min at 25°C to permeabilize the outer membrane, after which cells were resedimented by centrifugation and resuspended in the original volume of HNP (protein content of 10 mg/ml). TPP^+ distribution between the medium and the cytoplasm was determined by recording the external (medium) concentration of TPP^+ with a TPP^+ -selective electrode (Lolkema *et al.*, 1983). Measurements were performed in a compartment, thermostated at 30°C. Routinely, the measurement compartment was filled with 1.8 ml HNP and 4.0 μ M of TPP^+ , to which 200 μ l of EDTA-treated cells were added. In control experiments, 20 μ M nigericin (Sigma) was added to convert the proton gradient (ΔpH) into a measurable electrochemical potential ($\Delta\psi$). The binding constant of TPP^+ to cellular components was calculated from the electrode response upon addition of 200 μ l of cell suspension that had been treated for 30 min at 30°C with 1% toluene and 1% butanol. The fractional internal volume of intact cells (x) was taken as 3 μ l/mg of protein, as described by Lolkema *et al.* (1983).

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