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A *Salmonella* protein that is required for resistance to antimicrobial peptides and transport of potassium

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The ability of invading pathogens to proliferate within host tissues requires the capacity to resist the killing effects of a wide variety of host defense molecules. *sap* mutants of the facultative intracellular parasite *Salmonella typhimurium* exhibit hypersensitivity to antimicrobial peptides, cannot survive within macrophages *in vitro* and are attenuated for mouse virulence *in vivo*. We conducted a molecular genetic analysis of the *sapG* locus and showed that it encodes a product that is 99% identical to the NAD^+ binding protein TrkA, a component of a low-affinity K^+ uptake system in *Escherichia coli*. SapG exhibits similarity with other *E.coli* proteins implicated in K^+ transport including KefC, a glutathione-regulated efflux protein, and Kch, a putative transporter similar to eukaryotic K^+ channel proteins. *sapG* mutants were killed by the antimicrobial peptide protamine in the presence of both high and low K^+, indicating that protamine hypersensitivity is not due to K^+ starvation. Strains with mutations in *sapG* and either *sapJ* or the *sapABCDF* operon were as susceptible as *sapG* single mutants, suggesting that the proteins encoded by these loci participate in the same resistance pathway. SapG may modulate the activities of SapABCDF and SapJ to mediate the transport of peptides and potassium.

Key words: antimicrobial peptide/potassium transport/ *Salmonella*/virulence

Introduction

The ability of a microbe to prosper within animal host environments requires the capacity to synthesize nutrients not available from host tissues and to avoid or resist killing by toxic compounds (Garber, 1956). These compounds include several families of short, cationic peptides with antimicrobial activity which have been isolated from a wide range of animal species, including vertebrates and invertebrates (Boman, 1991; Zasloff, 1992). While these peptide families do not exhibit primary amino acid sequence identity, they share the capacity to adopt amphipathic structures and to generate voltage-gated channels in artificial membranes (Westerhoff *et al.*, 1989; Kagan *et al.*, 1990; Cruciani *et al.*, 1991). It has been postulated that microbial killing may result from the generation of such channels in the bacterial membrane through which essential metabolites would leak out of the cell.

Our laboratory has been interested in the mechanisms used by pathogens to resist the activity of different cationic peptides. We are using the facultative intracellular parasite *Salmonella typhimurium* as a model microorganism because it causes infections in a wide variety of animal species (Brenner, 1992) known to produce antimicrobial peptides, including insects, amphibians and mammals, and during the course of infection *Salmonella* resides in host tissues rich in these molecules (Groisman and Saier, 1990). One of the best characterized families of antimicrobial peptides are the defensins, arginine- and cysteine-rich cyclic peptides produced in both phagocytic and epithelial cells of several mammals (Lehrer *et al.*, 1993). Resistance to defensins is required for *Salmonella* pathogenicity, because mutants which are hypersusceptible to these peptides are unable to survive within macrophages *in vitro* and are attenuated for mouse virulence *in vivo* (Fields *et al.*, 1989; Groisman *et al.*, 1992). Moreover, in experimental infections of rabbit neutrophils, *Salmonella* has been shown to reside in vacuolar compartments rich in defensins, regardless of the opsonin used for uptake of the microbe (Joiner *et al.*, 1989). In mice, defensins have been purified from intestinal epithelial cells (Eisenhauer *et al.*, 1992; Ouellette *et al.*, 1992), but could not be detected in neutrophils (Eisenhauer and Lehrer, 1992). The antimicrobial properties of murine macrophages have been ascribed to histone and histone-like molecules harbored within cytosolic granules (Hiemstra *et al.*, 1993).

Using the histone-like protein protamine as a model peptide, we isolated a collection of *sap* (sensitive to antimicrobial peptides) mutants of *S.typhimurium* and identified eight distinct peptide resistance loci scattered around the genome (Groisman *et al.*, 1992). Our experiments demonstrated that resistance to protamine is required for mouse virulence and that multiple resistance mechanisms operate in *Salmonella*. One of the identified loci encodes the PhoP/PhoQ two-component regulatory system, which controls expression of genes required for intramacrophage survival and mouse virulence (García-Véscovi *et al.*, 1994), indicating that peptide resistance is transcriptionally regulated. Another *sap* locus, the *sapABCDF* operon (Parra-Lopez *et al.*, 1993), encodes the components of a novel peptide transporter that belongs to the 'ATP-binding cassette' (ABC) family (Higgins, 1992), also known as traffic ATPases (Doige and Ferro-Luzzi Ames, 1993). 'ABC' transporters participate in a variety of physiological functions in both prokaryotes and eukaryotes, including the detoxification of noxious compounds and the transport of ions. For example, the *S.typhimurium* SapABCDF transporter confers resistance to small cationic peptides (Parra-Lopez *et al.*, 1993),

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DrrAB protects *Streptomyces* from daunomycin and doxorubicin (Guilfoile and Hutchinson, 1991) and the mammalian Mdr P-glycoprotein pumps chemotherapeutic agents out of cancer cells (Gottesman and Pastan, 1993). An additional function has been attributed to Mdr: expression of P-glycoprotein in certain cell lines generates volume-regulated chloride-selective channels (Valverde *et al.*, 1992).

In this report, we describe the molecular genetic characterization of the *Salmonella sapG* locus. *Salmonella typhimurium sapG* mutants are attenuated for virulence in mice and are highly susceptible to the antimicrobial peptides protamine and melittin and to crude extracts from human neutrophil granules (Groisman *et al.*, 1992). Here, we establish the identity of SapG with a novel family of NAD^+ binding proteins required for both peptide resistance and K^+ transport and show that protamine hypersensitivity is not due to K^+ starvation and that membrane localization of SapG requires the presence of the SapJ peptide resistance determinant. Our data suggests that SapG may control other Sap proteins directly involved in transport activities.

Results

Cloning and expression of sapG

To investigate the peptide resistance determinant(s) defined by the MudJ insertion in the *sapG* strain EG1205, we cloned and analyzed wild-type and mutant DNA corresponding to this region of the genome. First, we isolated a DNA fragment containing the MudJ−*Salmonella* joint by preparing a genomic library from EG1205 and selecting for kanamycin (MudJ encoded)- and ampicillin (vector encoded)-resistant clones. One clone harbored plasmid pEG6009 with a 10 kb insert consisting of 2.9 kb of the left end of MudJ and 7.1 kb of *Salmonella* DNA adjacent to it. Sequence analysis of 1.7 kb of pEG6009 revealed the presence of a long open reading frame (ORF) immediately preceding MudJ. To clone the wild-type allele of *sapG*, we PCR amplified a 690 bp fragment internal to the ORF and used the labeled fragment to screen a plasmid library by colony hybridization. Two hybridizing clones harbored plasmids with inserts that included several restriction fragments in common with each other and with the clones containing the MudJ−*Salmonella* joint. Both plasmids, pEG7170 and pEG7171 (Figure 1A), conferred a protamine-resistant phenotype to CP1205, a kanamycin-sensitive, ampicillin-resistant derivative of EG1205. We used CP1205 because the wild-type library was generated with the vector Mud5005 (Groisman and Casadaban, 1986), which encodes resistance to kanamycin, like the MudJ element in strain EG1205. Then we tested several subclones harboring pEG7170-derived DNA segments for their ability to confer protamine resistance to strain EG1205 and localized *sapG* to the 1.8 kb *Apo*I fragment in plasmids pEG7157 and pEG7158 (Figure 1A). These plasmids harbor the same 1.8 kb insert in opposite relative orientations, suggesting that both coding and promoter regions were present. Protamine resistance was destroyed upon insertion of a *kan* cassette in either orientation at the unique *Nru*I site of pEG7157 (plasmids pEG7177 and pG7178; Figure 1A).

To identify the product(s) encoded by the *sapG* locus, we prepared extracts from *Escherichia coli* JM109 cells

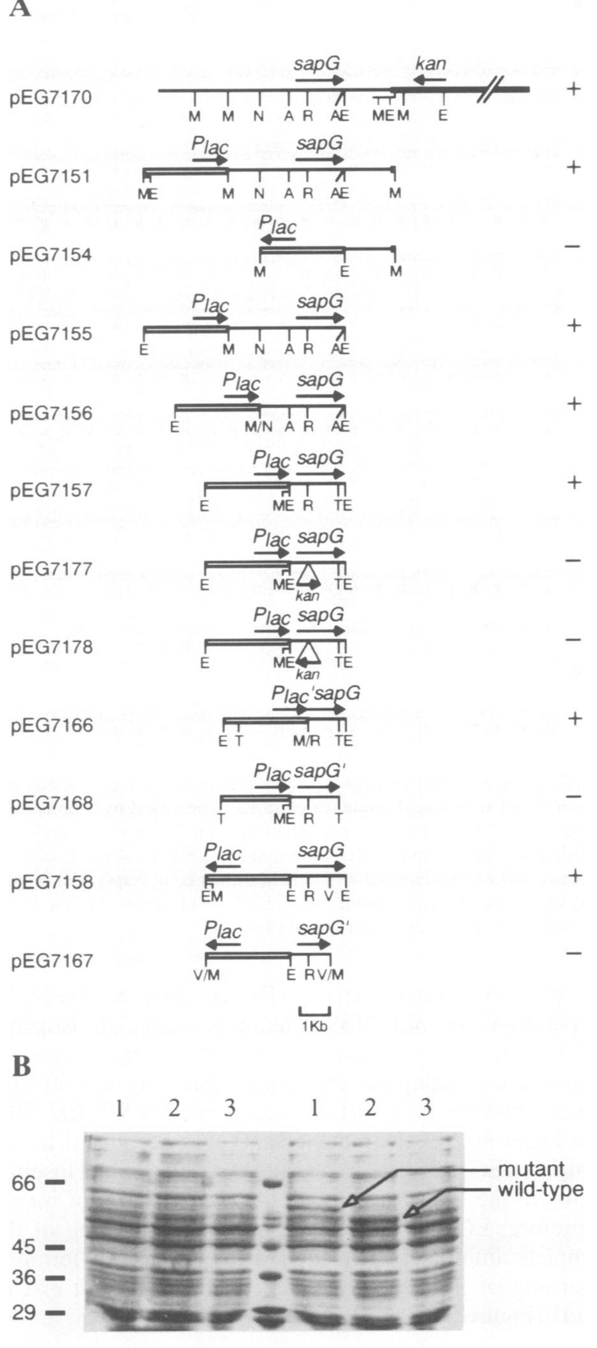

Fig. 1. Cloning and expression of *sapG*. **(A)** Physical maps and complementation data for *sapG*-containing plasmids. Dark and open bars correspond to Mud5005 and pUC19 vector sequences respectively and the thin line corresponds to *S.typhimurium* chromosomal DNA. The ability of the various plasmids subclones to complement the protamine susceptibility defect of EG1205 is indicated: +, growth; −, no growth. Restriction sites are abbreviated as follows: A, *Apo*I; E, *Eco*RI; M, *Sma*I; N, *Nae*I; R, *Nru*I; T, *Aat*II; V, *Eco*RV. **(B)** Expression of *sapG*. Lysates were prepared and processed as described in Materials and methods from JM109 cells harboring the following plasmids: 1, pEG7159, with *sapG1205* (Figure 4); 2, pEG7157, with wild-type *sapG*; 3, pUC19 as control; + and − indicate addition or no addition of IPTG. Numbers indicate molecular weight in kDa.

harboring plasmid pEG7157, with the *sapG* gene downstream of the *lac* promoter in pUC19. Extracts from IPTG-induced cells had a 50 kDa protein that was absent from

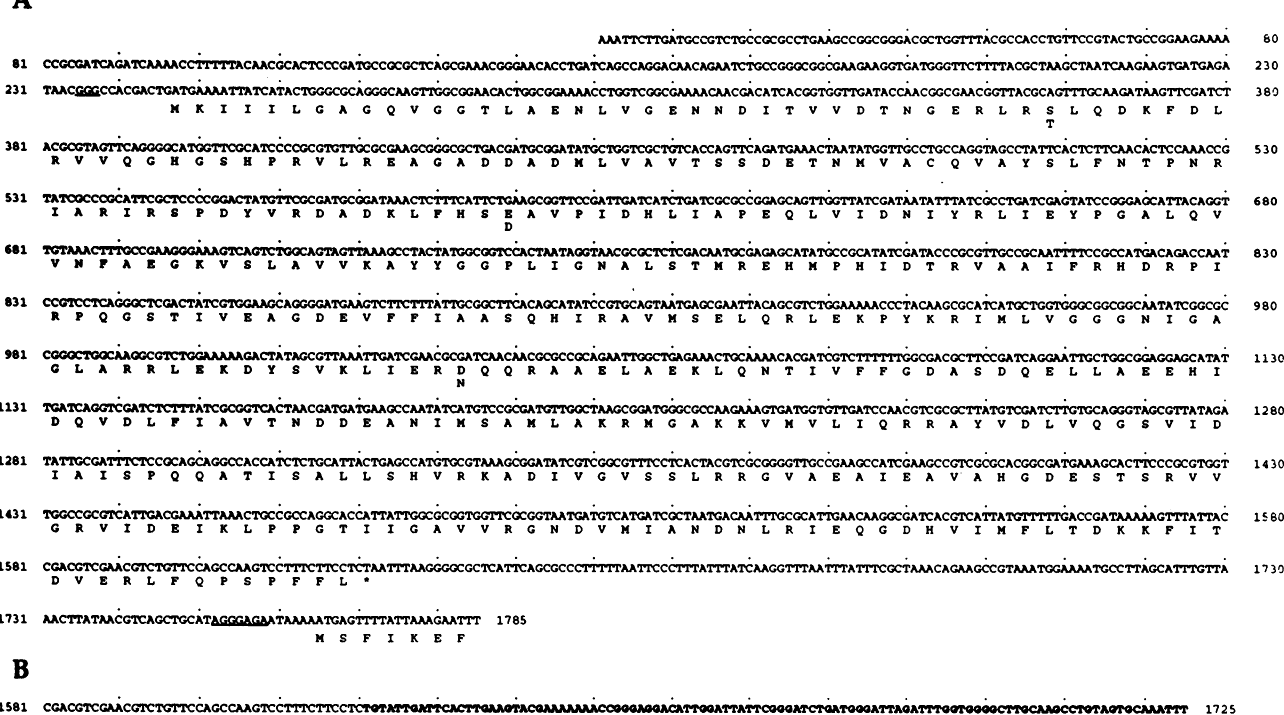

Fig. 2. DNA sequence of wild-type and mutant *sapG*. (**A**) Nucleotide sequence of the 1785 bp *Apo*I fragment of plasmid pEG7157 harboring *sapG*. The deduced amino acid sequence of *sapG* is preceded by a potential ribosome binding site (underlined). Downstream of *sapG* is the beginning of the *mscL* gene preceded by a potential ribosome binding site (underlined). The nucleotide sequence reported in this paper has been deposited in the EMBL Data Library under accession number X80501. Amino acid residues of the *E.coli* TrkA protein that differ from *S.typhimurium* SapG are indicated below the deduced amino acid sequence of SapG. (**B**) Nucleotide sequence of joint region and deduced amino acid sequence of predicted product corresponding to mutant *sapG1205*. MudJ-derived DNA and the deduced amino acid sequence of an additional 31 residues encoded by the left end of MudJ are indicated in bold face.

the pUC19 control extract (Figure 1B). Surprisingly, preparations of pEG7159-containing cells, an isogenic construct carrying the mutant *sapG1205* allele (Figure 4), harbored a polypeptide that was larger than the 50 kDa protein present in wild-type *sapG* extracts (Figure 1B). The larger size of the mutant SapG was accounted for by analyzing EG1205 DNA: the MudJ transposon had inserted between the last codon and the stop codon of *sapG*. Therefore, EG1205 harbored a product consisting of the complete amino acid sequence of SapG plus a C-terminal extension of 31 amino acids encoded by the left end of MudJ (Figure 2B).

Sequence analysis of SapG reveals identity with potassium transport proteins

The nucleotide sequence of the 1.8 kb segment from plasmid pEG7157 was analyzed to identify the determinant(s) responsible for peptide resistance. We found a single open reading frame in one of the strands encoding a product of 458 amino acids (predicted mol. wt 50 368; Figure 2A), in close agreement with that observed in the expression experiments (Figure 1B). That this open reading frame is likely to correspond to the peptide resistance determinant mutated in EG1205 is based on: (i) its codon usage pattern, which appears typical of other moderately expressed *S.typhimurium* genes; (ii) the extensive sequence similarity of SapG with other proteins in the databases; (iii) the small size (≤85 residues) of all remaining open reading frames within the analyzed fragment; (iv) the presence of stop codons in all three reading frames downstream of *sapG*. Analysis of SapG revealed that it consists of two tandemly arrayed halves that exhibit 22% identity (51% similarity) with each other (Figure 3A), a structure which might have arisen by gene duplication.

A search of the sequence databases (GenBank release 79) for SapG homologs revealed identity with proteins implicated in the transport of potassium. Using the program TFASTA, we found that SapG was 99% identical (only three amino acid substitutions in 458 residues) to TrkA, an NAD^+ binding protein that is part of a low-affinity potassium uptake system of *E.coli* (Schlösser *et al.*, 1993; Figure 2A). None of the three amino acid differences between the *E.coli* and *Salmonella* proteins mapped to residues corresponding to the predicted NAD^+ binding sites, which are shared with several dehydrogenases (Figure 3A). Several open reading frames of unknown function exhibiting sequence similarity to SapG were detected in the genomes of both Eubacteria and Archaebacteria, suggesting that SapG may be required for a common cellular physiological function (Figure 3A). Inspection of the databases using the program BLAST revealed similarities with other K^+ transport proteins of *E.coli*. A stretch of 106 amino acids in the N-terminal half of SapG exhibited 28% identity (47% similarity) with KefC, a glutathione-regulated potassium efflux system (Munro *et al.*, 1991; Figure 3B), and a 54 residue segment in the C-terminal half of SapG showed 29% identity (51% similarity) with Kch1 (Figure 3B), a putative K^+ trans-

A

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SapG      1 MKIIILGAGQ VGGTLAENLV GENN.DITVV DTNGERLRSL QDKFD.LRVV  48
SapG    233 KRIMLVGGGN IGAGLARRLE KD..YSVKLI ERDQQRAAEL AEKLQNTIVF 280
MmTrkA    1 MKAVIIGAGE VGYHIAKAL. SPKN.DVVII DKDEEAAKR. ADELD.VLVI  46
MmTrkA  230 TRILLIGCGI VGMYLAKLID KEENADLRII EHSKSRCIEV AEILENALVL 280
AcOrf2    1 MKVVICGAGQ VGFGIAERLA SEQN.DVSIV DASPRRIQIA TDQLD.VRGV  48
ScOrfr    1 VHIVIMGCGR VGSALAQALE QQGH.TVAVI DRDPTSFRRL GSSFG.GRRV  48
                  * *   *   *
BsDha   167 GKVTIIGGGV VGTNAAKMAV                                 186

SapG     49 QGHGSHPRVL REAGADDADM LVAVTSSDET NMVACQVAYS LFNTPNRIAR  98
SapG    281 FGDASDQELL AEEHIDQVDL FIAVTNDDEA NIMSAMLAKR MGAKKVMVLI 330
MmTrkA   47 EGNGANAEIL SR.VLQNADL LVAVTGVDEV NIVACMTAKL IMKNKNGWKD  95
MmTrkA  281 NGDGTDVSLL REENIEDMDV VVAVTDSDEK NLLCSLLAKQ LGAKKVIARA 330
AcOrf2   49 VGHGSHPDVL ARAGIEQADM LIAVTLHDEV NMVACQVGHS LFNVPTSPAS  98
ScOrfr   49 TGIGFDQDTL REAGIEEAGA FAAVSSGDNS NIIAARVARE MFGVENVAAR  98
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B

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SapG      1 MKIIILGAGQ VGGTLAENLV GENNDITVVD TNGERLRSLQ DKFDLRVVQG  50
KefC    400 PRVIIAGFGR FGQITGRLLL SSGVKM.VVL DHDPDHIETL RKFGMKVFYG 449

SapG     51 HGSHPRVLRE AGADDADMLV AVTSSDETNM VACQVAYSLF NTPNRIARIR 100
KefC    450 DATRMDLLES AGAAKAEVLI NAIDDPQTNL QLTEMVKEHF PHLQIIARAR 499

SapG    233 KRIMLVGGGN IGAGLARRLE KDYSVKLIER DQQRAAELAE KLQNTIVFFG 282
Kch1    290                                                NADVIPG 296

SapG    283 DASDQELLAE EHIDQVDLFI AVTNDDEANI MSAMLAKRMG AKKVMVLIQR 332
Kch1    297 DSNDSSVLKK AGIDRCRAIL ALSDNDADNA FVVLSAKDMS SDVKTVLAVS 346
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Fig. 3. Comparison of SapG with related proteins. (**A**) Alignment of N- and C-terminal halves of SapG with each other as well as with the deduced amino acid sequences of open reading frames in genomes of *M.mazei* (MmTrkA; Macario *et al.*, 1993), *A.caulinodans* (AcOrf2; Pawlowski *et al.*, 1991) and *S.coelicolor* (ScOrfr; Narva and Feitelson, 1990). The region of similarity between the predicted NAD^+/NADH binding site and that of a *Bacillus subtilis* dehydrogenase (BsDha) is also shown; * indicates conserved residues in predicted NAD^+ binding site. (**B**) Alignment of amino acid sequence of SapG with *E.coli* proteins implicated in K^+ transport: the glutathione-regulated potassium efflux KefC (Munro *et al.*, 1991) and the Kch1 protein with sequence identity to voltage-activated K^+ channels of eukaryotes (Milkman, 1994). Alignments were performed using a combination of the programs TFASTA (average match, 0.54; average mismatch, −0.396) and PILEUP (gap weight, 3.0; gap length weight, 0.1) from GCG. Identical or similar amino acids within each comparison are highlighted and grouped as follows: AST, DEQ, ILMV, RK, FYW.

porter similar to a group of eukaryotic K^+ channel proteins (Milkman, 1994). While both KefC (Munro *et al.*, 1991) and Kch1 (Milkman, 1994) have several predicted transmembrane domains, the identity with SapG was localized to their C-terminal cytoplasmic regions. Downstream of the *sapG* coding region we identified the beginning of an ORF preceded by a putative ribosome binding site (Figure 2A). Six out of the seven residues of this OFR were identical to MscL, a mechanosensitive channel of *E.coli* (Sukharev *et al.*, 1994). *mscL* is 133 bp downstream of *trkA* in *E.coli* and 144 bp downstream of *sapG* in *S.typhimurium*.

Molecular genetic analysis of the sapG locus

Because the complete amino acid sequence of SapG was present in mutant EG1205, the observed protamine hypersusceptibility could be ascribed to either an inactive SapG (due to the C-terminal extension) or result from polarity of the MudJ insertion on an ORF downstream of *sapG* (Figure 2A). We ruled out the second possibility by demonstrating that a plasmid carrying the PCR amplified coding region of *sapG* driven by the *lac* promoter of pUC19 (plasmid pEG7193) could rescue protamine resistance in EG1205 (Figure 4). Then, we examined whether the N- and C-terminal halves of SapG (which are 51% similar) could complement EG1205 when expressed alone. Protamine resistance was rescued by plasmid pEG7196 (carrying the PCR generated C-terminal half of *sapG*), but not by pEG7194 (which harbored the N-terminal half of *sapG*). These results indicated that the two halves of the molecule were not functionally equivalent and that the C-terminal half of *sapG* was sufficient for protamine resistance. However, rescue of EG1205 could also be explained by intragenic complementation.

To evaluate these hypotheses, we constructed null chromosomal mutants by transferring the *sapG*::*kan* mutations in plasmids pEG7177 and pEG7178 (Figure 1A) to the *S.typhimurium* chromosome. The resulting strains, RL1205-4 and RL1205-6, with the *kan* cassette in both possible orientations and encoding only the 106 N-terminal amino acids of *sapG* (Figure 4), were sensitive to protamine. These mutants could be rescued by pEG7157 (with full-length *sapG*), but not by pEG7196 (with the C-terminal half of SapG). These results are consistent with SapG being an oligomer and with rescue of EG1205 resulting from complementation of a C-terminal altered SapG by an N-terminal-deleted SapG and they are in agreement with intragenic complementation observed with *E.coli trkA* (cited in Bakker, 1993b). To investigate whether SapG could oligomerize, we ran extracts from *E.coli* cells that overexpressed wild-type SapG in a gel filtration column and tested individual fractions for the presence of SapG using an ELISA assay with anti-TrkA antibodies. Peaks of activity were detected with fractions corresponding to molecular weights of about 50 kDa, 150 kDa and 670 kDa (Figure 5). While these results are consistent with SapG homo-oligomerization, they can also be interpreted as SapG forming complexes with other proteins. The SapG1205 mutant protein behaved in an identical fashion, indicating that the C-terminal extension was altering a property other than oligomerization.

Strain	Plasmid structure	Predicted products	Protamine resistance
14028s	na	458 aa	+
EG1205	na	489 (458+31) aa	-
RL1205-6	na	136 (106+30) aa	-
EG1205 /pEG7157	Plac sapG; E T ME R TE		+
RL1205-6 /pEG7157	Plac sapG; E T ME R TE		+
EG1205 /pEG7159	Plac sapG; E T ME R TE		-
EG1205 /pEG7193	Plac sapG; E HR TE	458 aa	+
EG1205 /pEG7194	Plac sapG'; B HRB	235 aa	-
EG1205 /pEG7196	Plac'sapG; E B TE	223 aa	+
RL1205-6 /pEG7196	Plac'sapG; E B TE		-

Fig. 4. Molecular genetic analysis of the *sapG* locus. Physical and genetic maps of *sapG*-containing plasmids and predicted structures of the encoded *sapG* gene products. The complementation phenotype of the various plasmids when introduced in the different chromosomal *sapG* mutants is indicated. Restriction sites were abbreviated as described in Figure 1; B, *Bam*HI; H, *Hin*dIII.

SapG is required for protamine resistance and potassium transport

The identity of SapG with a component of the Trk potassium uptake system raised the possibility that protamine sensitivity could be an indirect result of impaired growth associated with K^+ starvation and that protamine resistance was directly related to K^+ transport through Trk. To evaluate these hypotheses it was necessary to establish conditions where the growth rate of the *sapG* mutant was normal and the Trk activity of the wild-type strain was repressed. *Escherichia coli* (and presumably other enteric bacteria) harbors several K^+ uptake systems (Bakker, 1993a), including the high-affinity, low-capacity Kdp (Siebers and Altendorf, 1993) and the low-affinity, high-capacity Trk (Bakker, 1993b), which function at micromolar and millimolar levels of K^+ respectively, and the Kup system, which has been implicated in the transport of both cesium and potassium (Bakker, 1993b). We presume the presence of Kdp, Trk and Kup activities in *S.typhimurium* based on the presence of hybridizing DNA sequences in this species (Walderhaug *et al.*, 1989; Bakker, 1993b; Parra-Lopez *et al.*, 1993).

The *sapG* mutants EG1205 and RL1205-6 showed impaired growth in media containing 10 mM K^+ with either glucose or glycerol as sole carbon sources. For example, in a glycerol minimal medium the doubling times for the wild-type and EG1205 strains were 62 and 75 min respectively. The impaired growth rate was presumably due to repression of the Kdp system and a slower rate of uptake through Kup. On the other hand, wild-type and *sapG* mutants had identical growth rates of 60 and 65 min when grown with glycerol and 35 μM or 115 mM K^+, respectively. A concentration of 35 μM K^+ is sufficiently low to allow expression of the Kdp system, which could compensate for K^+ uptake deficiencies in the Trk transporter, whereas a concentration of 115 mM K^+ is high enough to allow normal growth rates even in *kdp trk* mutants of *E.coli* (Rhoads *et al.*, 1976).

The *sapG* mutant EG1205 exhibited hypersensitivity to protamine in media containing either 35 μM or 115 mM K^+. Because EG1205 grew at wild-type rates at both concentrations of K^+, these results argue that protamine sensitivity was not due to K^+ starvation. When incubated in the presence of 115 mM K^+ and 10 μg/ml protamine, EG1205 showed a level of hypersensitivity similar to that seen with 3 μg/ml in 35 μM K^+ medium (Figure 6). The 3-fold higher concentration of protamine was required in the 115 mM K^+ medium because of competition between K^+ and protamine for binding sites in the bacterial outer membrane. This effect was not specific for K^+, as excess Na^+ or Mg^{2+}, supplied as 115 mM NaCl or 20 mM $MgSO_4$, also conferred increased resistance to protamine. Furthermore, the wild-type strain was resistant to protamine under all K^+ concentrations tested, including condi-

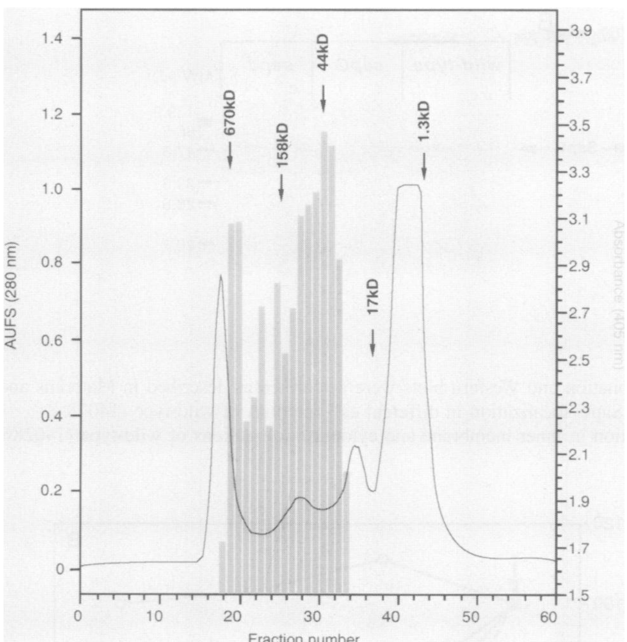

Fig. 5. Oligomerization of SapG. Extracts corresponding to *E.coli* cells overproducing *S.typhimurium* SapG were prepared and analyzed as described in Materials and methods. Bars represent reactivity of anti-TrkA antiserum against different chromatographic fractions. Tracing corresponds to the protein profile of the chromatographic run. Arrows indicate the position of elution of protein size markers (thyroglobulin, 670 kDa; bovine γ-globulin, 158 kDa; ovalbumin, 44 kDa; myoglobin, 17 kDa; vitamin B-12, 1.35 kDa).

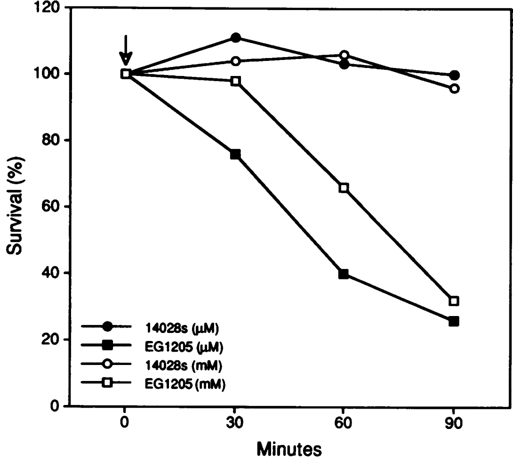

Fig. 6. Effect of K^+ concentration on protamine susceptibility of wild-type (14028s) and *sapG* (EG1205) *S.typhimurium*. Log phase cells were incubated at 37°C in a MES-buffered minimal medium, pH 6.3. Cells were incubated with 10 mM DL-lactate, 3 μg/ml protamine and 35 μM K^+ (closed symbols) or with 10 mM glycerol, 10 μg/ml protamine and 115 mM K^+ (open symbols). Protamine was added at the arrow. Values are the average of at least three trials.

tions where the Trk system was not fully active. This implies that K^+ transport through Trk *per se* is not required for protamine resistance.

Interaction of SapG with other Sap proteins

The SapG hydropathy profile predicts a cytoplasmic protein devoid of the long hydrophobic stretches typically found in integral membrane proteins (data not shown). However, in Western blot experiments of different subcellular fractions prepared from the wild-type strain, SapG localized to the inner membrane (Figure 7A). To identify the membrane protein(s) that SapG may associate with, we tested the subcellular localization of SapG in strains defective in *sap* loci known to encode integral membrane proteins. SapG exhibited a wild-type localization pattern in a strain in which the *sapABCDF* operon had been deleted (Parra-Lopez *et al.*, 1993; Figure 7A), however, in a *sapJ* mutant about half of the SapG remained in the cytoplasm (Figure 7B). This suggests that SapG might interact with SapJ, a protein with 10 predicted transmembrane domains (E.A.Groisman and M.T.Baer, unpublished results). As expected, no reactivity was detected in either the cytoplasm or inner membrane fractions of *sapG* mutants (Figure 7).

Sap proteins that interact are likely to participate in the same pathway of peptide resistance and one would predict that a strain with mutations in the corresponding *sap* loci would not be any more sensitive than strains with single *sap* mutations. Indeed, we found that strains EG6571 (*sapG sapJ*) and EG6572 (*sapG sapD*) exhibited protamine susceptibility comparable to that of strains carrying single mutations in either *sapG*, *sapJ* or the *sapABCDF* operon (Figure 8A). We investigated whether PhoP, a transcription factor that controls resistance to several antimicrobial peptides, including protamine (Groisman *et al.*, 1992), was part of the same resistance pathway as the *sap* loci listed above. Survival of a *sapG phoP* mutant was decreased 2- to 4-fold relative to that observed with either *sapG* or *phoP* strains (Figure 8B). Likewise, heightened susceptibility was observed with *sapJ phoP* and *sapC phoP* mutants relative to the isogenic strains with single mutations (data not shown). Cumulatively, these results are consistent with SapG, SapJ and SapABCDF being part of the same resistance pathway and PhoP controlling expression of different resistance determinants.

Discussion

Animals produce a variety of microbicidal compounds that constitute a barrier to infection by organisms of the normal flora or opportunistic pathogens (Du Pasquier, 1992). The battery of noxious compounds includes short cationic peptides with potent microbicidal activity against a broad range of microbes (Boman, 1991; Zasloff, 1992). The target of these antimicrobial peptides is postulated to be the cytoplasmic membrane of Gram-positive and Gram-negative bacteria. These peptides would insert in the lipid bilayer to generate voltage-gated channels resulting in leakage of essential cellular components and death of the microbe. Successful pathogens have evolved different mechanisms to evade the microbicidal effects of these molecules. For example, the facultative intracellular pathogen *S.typhimurium* harbors several proteins which enable it to resist killing by these peptides (Groisman, submitted) and strains with mutations in the corresponding genes are avirulent (Groisman *et al.*, 1992).

In this paper we characterize the protamine resistance determinant SapG and establish its identity with TrkA, an NAD^+ and NADH binding protein of *E.coli* implicated in K^+ transport (Schlösser *et al.*, 1993). It has been proposed that the Trk K^+ uptake system is composed of

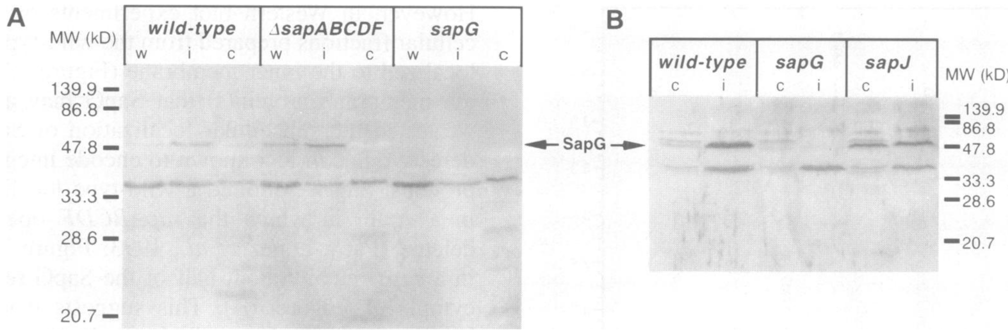

Fig. 7. Subcellular localization of SapG in wild-type and *sap* mutants. Fractionation and Western blots were performed as described in Materials and methods. w, whole membrane fraction; i, inner membrane; c, cytoplasm. (**A**) SapG localization in different cell fractions of wild-type (14028s), Δ*sapABCDF* (EG6501) and *sapG* null (RL1205-6) strains. (**B**) SapG localization in inner membrane and cytoplasmic fractions of wild-type (14028s), *sapG* null (RL1205-6) and *sapJ* (EG1207) strains.

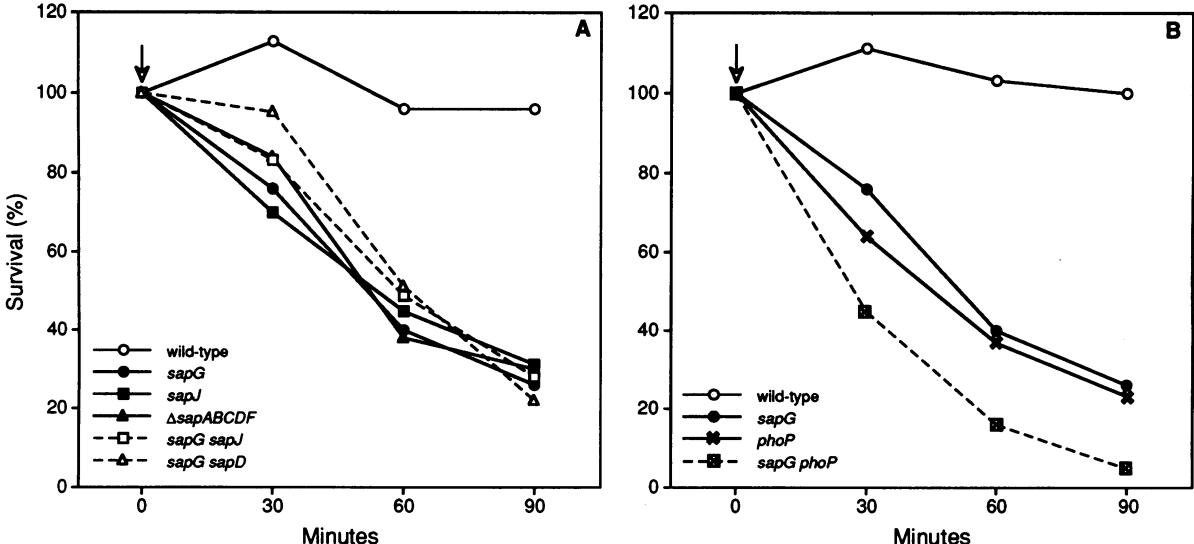

Fig. 8. Comparison of protamine susceptibility among wild-type and protamine-sensitive mutants. Log phase cells were incubated at 37°C in minimal medium containing 10 mM DL-lactate, 3 μg/ml protamine and 35 μM K^+, pH 6.3. Protamine was added at the arrow. Values are the average of at least three trials. (**A**) Comparison of wild-type (14028s), *sapG* (EG1205), *sapJ* (EG1207), Δ*sapABCDF* (EG6501), *sapG sapJ* (EG6571) and *sapG sapD* (EG6572) strains. (**B**) Comparison of wild-type (14028s), *sapG* (EG1205), *phoP* (EG6587) and *sapG phoP* (EG5195) strains.

TrkA associated with either TrkH and TrkE or with TrkG (Dosch *et al.*, 1991; Bakker, 1993b). We found that *sapG* mutants exhibited slower growth rates at millimolar K^+ concentrations, providing the first physiological evidence for the presence of a functional Trk system in *Salmonella*. We would like to suggest a regulatory role for SapG based on the region of similarity it shares with other proteins implicated in K^+ transport (Figure 3B). This region could correspond to a ligand binding site(s) that allows a bacterium to coordinate the activities of the K^+ efflux KefC system (Munro *et al.*, 1991), the K^+ uptake system Trk and Kch1, a putative K^+ transporter with structural and sequence similarities to eukaryotic K^+ channel proteins (Milkman, 1994). The regions of SapG identity in KefC and Kch1 localize to their C-terminal cytoplasmic domains, which in the eukaryotic voltage-gated channels correspond to ligand binding sites.

Given that K^+ transport through the Trk system requires a functional SapG and that certain antimicrobial peptides cause K^+ leakage (e.g. insect defensins in *Micrococcus luteus* (Cociancich *et al.*, 1993), we investigated whether protamine hypersensitivity could result from the inability of *sapG* mutants to compensate for the leaked K^+, leading to K^+ starvation. However, we have ruled out this possibility, since the *sapG* mutants displayed protamine hypersensitivity even in K^+ concentrations where they exhibited no growth defects (Figure 6). Moreover, the wild-type strain remained resistant under conditions where the Trk system was not expected to be active. Peptide susceptibility could be associated with the inability of *sapG* mutants to maintain a physiological electrical membrane potential, thereby facilitating the activity of protamine, or to inhibit the activity of protamine. The NAD^+/NADH binding capacity demonstrated with the *E.coli* homolog of SapG (Schlösser *et al.*, 1993) suggests that SapG could be involved in sensing reducing power and regulating other Sap proteins, such as SapJ and the SapABCDF peptide transporter.

Consistent with participating in the same resistance pathway, strains with mutations in *sapG* and either *sapJ* or *sapD* were no more susceptible to protamine than strains with single mutations in these loci. Furthermore,

the inner membrane localization of SapG required an intact SapJ (Figure 7B), a protein with 10 predicted transmembrane domains (E.A.Groisman and M.T.Baer, unpublished data) that is 97% identical to TrkH (E.Bakker, personal communication). Interestingly, the *sapABCDF* operon corresponds to the *trkE* locus of *E.coli* (C.Parra-Lopez and E.A.Groisman, unpublished data), indicating that the same set of genes were identified in two independent screenings: *trkA*, *trkH* and *trkE* as components of the Trk low-affinity K^+ uptake system and their *Salmonella* homologs based on their role in resistance to protamine. The only *trk* component not recovered in *Salmonella* as a *sap* gene is *trkG*, because it is encoded by a cryptic prophage only present in certain strains of *E.coli* (Schlösser *et al.*, 1991).

We would like to propose that SapG, SapJ and the SapABCDF transporter function as a complex to mediate both peptide and K^+ transport. SapABCDF is an 'ABC' transporter most closely related to systems involved in peptide uptake (Parra-Lopez *et al.*, 1993) and would constitute the peptide 'pore' of this complex. SapJ/TrkH could be the K^+ 'pore', since the Trk system can function with just SapG/TrkA and TrkG (Bakker, 1993b), which is 42% identical to TrkH (Schlösser *et al.*, 1991). SapG could act to coordinate the peptide and K^+ transport functions of the complex. However, alternative models (e.g. SapJ and SapABCDF forming a single pore for both K^+ and peptides) cannot be ruled out at the present time. In this regard, the Sap complex is reminiscent of the human P-glycoprotein: Mdr has been shown to mediate peptide transport in yeast (Raymond *et al.*, 1992) and to influence the activity of chloride channel currents in mammalian cell lines (Gill *et al.*, 1992). The peptide transport activities of this complex would be responsible for peptide resistance and contribute to *Salmonella*'s ability to survive within macrophages *in vitro* (C.Parra-Lopez and E.A.Groisman, unpublished data) and to cause a lethal infection in mice *in vivo* (Groisman *et al.*, 1992). The presence of SapG, SapJ and SapABCDF homologs in *E.coli* and other enteric bacteria suggests that this system participates in essential physiological activities, such as K^+ uptake and osmoadaptation, common to several bacterial species.

Materials and methods

Bacterial strains, plasmids and growth media

Mutant strains of *S.typhimurium* were isogenic derivatives of the wild-type peptide-resistant strain 14028s. These mutants are designated as follows: CP1205, *sapG*::Mud1-8; EG1205, *sapG*::MudJ; EG1207, *sapJ*::MudJ; EG6501, Δ*sapABCDF*::*kan*; EG5195, *sapG*::MudJ *phoP*::Tn*10*; EG6571, *sapG*::Mud1-8 *sapJ*::MudJ; EG6572, *sapG*::Mud1-8 *sapD*::MudJ; EG6587, *phoP*::Tn*10*; RL1205-4, *sapG*::*kan*, RL1205-6, *sapG*::*kan*; *E.coli* JM109: F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/*e14*$^-$ (McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* (r_k^- m_k^+) *relA1 supE44 recA1* (Yanisch-Perron *et al.*, 1985). The chromosomal mutations in strains RL1205-4 and RL1205-6 were constructed as previously described (Groisman *et al.*, 1993) using pEG7157 derivatives harboring the *kan* 1.3 kb *Sma*I fragment from plasmid pUC4-KIXX (Pharmacia). The structure of *sapG* in the chromosomal mutants was verified by PCR-mediated amplification using two sets of *sapG* primers corresponding to DNA sequences on both sides of the *kan* insertion (data not shown). Additional mutants were constructed by phage P22-mediated transduction with selection for the antibiotic resistance markers in the transposon. Plasmid pUC19 (Yanisch-Perron *et al.*, 1985) was used for subcloning and pEG5005 (Groisman and Casadaban, 1986) for the construction of genomic libraries by the *in vivo* cloning procedure (Groisman, 1991). Plasmids pEG7193, pEG7194 and pEG7196 were constructed by the PCR amplification of *sapG* DNA followed by cloning into pUC19. To make pEG7193 we used primers 319 (5′-GCGGAATTCTTAGAGGAAGAAAGG-3′) and 320 (5′-CGC-AAGCTTAGGACTGATGAAAAT-3′); to make pEG7194 we used primers 320 and 321 (5′-TCGGATCCTCAGATGCGCTTGTAGG-3′); to make pEG7196 we used primers 319 and 323 (5′-TCGGATCCAA-GCGCATCATGCT-3′). LB (Miller, 1972) and K (Epstein and Kim, 1971) media were prepared as described. Kanamycin (Kan) was used at 40 μg/ml, ampicillin (Ap) at 50 μg/ml and tetracycline (Tc) at 10 μg/ml. Protamine (Calbiochem)-containing LB agar plates were prepared fresh to final concentrations of 0.5–1.5 mg/ml.

DNA biochemistry and molecular biological techniques

Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories Inc., Boehringer Mannheim Biochemicals or New England BioLabs Inc. and were used according to the manufacturer's specifications. The wild-type *sapG* gene was cloned from a genomic library prepared by the *in vivo* cloning technique using the Mud5005 mini-Mu replicon as described (Groisman, 1991). DNA was purified from host cells using reagents and midi-prep columns from QIAGEN Inc. The nucleotide sequence was determined by the dideoxy-nucleotide chain termination method using Sequenase Version 2.0 (USB), [α-^{35}S]dATP (Amersham), templates corresponding to plasmids pEG 7157 and pEG7159 and primers synthesized using the phosphoramidite method in a Cyclone Plus DNA synthesizer (Millipore), based on the sequence obtained. The DNA sequence presented in this paper was determined completely on both strands. Computer analyses were performed using the software packages GCG (University of Wisconsin Biotechnology Center, Madison, WI) and GeneWorks (Intelligenetics). We searched the sequence databases for protein similarities using the programs TFASTA and BLAST. PILEUP was used for optimal alignment of protein sequences.

Protein biochemistry and expression

Expression of wild-type and mutant versions of *sapG* was achieved using JM109 cells harboring plasmids pEG7157 and pEG7159 with the *sapG* gene (with its own regulatory region) behind the *lac* promoter of pUC19. Single colonies were used to inoculate pairs of 2 ml LB broth cultures containing Ap (50 μg/ml) and IPTG (0 or 1 mM). Cultures were grown at 37°C with shaking for 5 h and cells were adjusted to A_{600} = 0.7. Then, 1.5 ml aliquots were transferred to Eppendorf tubes, cells were spun down in a microfuge and the pellet was resuspended in 245 μl loading buffer. Samples were boiled for 5 min and centrifuged in a microfuge for 5 min before loading 30 μl onto a 10% SDS–PAGE gel. Gels were stained with Coomassie blue. Reagents for protein electrophoresis analysis were from Bio-Rad. When testing the oligomerization potential of SapG, a larger volume of JM109 cells harboring pEG7157 and pEG7159 was induced. We used 600 μl overnight cultures to inoculate 30 ml LB broth containing Ap (50 μg/ml) and 0.1 mM IPTG. Cells were grown at 37°C with shaking to A_{600} = 0.5. An aliquot was processed as described above and run in an SDS–PAGE gel to check for efficient induction of *sapG* (data not shown). The remaining cells were spun down, resuspended in 4 ml buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0, 150 mM NaCl) and frozen at −20°C overnight. Cells were thawed and sonicated using 15 s pulses until no visible release of proteins was detected. Then, cells were spun down at 9000 *g* at 4°C for 20 min and the supernatant was concentrated six times using a Centricon 10 (Amicon). An aliquot of total protein (1.2 mg) was loaded onto a Waters 300 SW gel filtration column and run on a Waters HPLC 625 LC. Using a flow rate of 0.35 ml/min, 60 fractions were collected and A_{280} was followed using a Waters 486 UV detector. Standards used corresponded to proteins of the following molecular weights: 670 kDa, 158 kDa, 44 kDa, 17.5 kDa and 1.35 kDa. To test for SapG reactivity, 96-well ELISA plates (Costar) were covered with individual fractions diluted 1:100 in bicarbonate coating buffer, pH 9.6. Plates were incubated overnight at 4°C for protein binding. Then they were blocked with a phosphate-buffered saline (PBS) solution containing 0.2 % gelatin for 1 h at 37°C with shaking. Anti-TrkA antibodies (a generous gift from E.Bakker, University of Osnabrück) were used at 1:3000 dilution in PBS containing 0.1% Tween 20 (Sigma). The second antibody was anti-rabbit IgG coupled to alkaline phosphatase (Promega). Washes were done in PBS containing 0.1% Tween 20. Hydrolysis of *p*-nitrophenyl phosphate (dissolved at 1 mg/ml in diethanol amine buffer, pH 9.8) was measured at A_{405} using a plate reader (Molecular Devices Inc.).

For cell fractionation experiments, bacteria were grown overnight in 20 ml LB broth (with antibiotic if needed) at 37°C. The following day cells were spun down and resuspended in 0.8 ml 20 mM Tris/20% sucrose. We then added 40 µl 0.1 M EDTA and 12 µl 15mg/ml lysozyme to the cell suspension and incubated on ice for 40 min. Then, 32 µl of 0.5 M magnesium chloride was added and cells were spun down at 10 000 r.p.m. The supernatant, containing mostly periplasmic contents, was separated from the cell pellet and stored away. The cell pellet was resuspended in 500 µl ice-cold 10 mM Tris, pH 8.0, and sonicated for 240 s with a 30 s rest. Next, cell debris was spun down at 8000 r.p.m. for 5 min. The resulting supernatant contained inner membranes, outer membranes and cytoplasm. Then the membranes were separated from the cytoplasm by centrifugation at 20 000 r.p.m. for 60 min. The pellet, consisting of both inner and outer membranes, was washed twice with 10 mM Tris, pH 8.0, to remove contamination by cytoplasm and resuspended in 30 µl water. An aliquot of the membrane fraction (15 µl) was mixed with 200 µl sarkosyl solution and incubated for 20 min at room temperature. Centrifugation at 20 000 r.p.m. for 90 min separated inner (supernatant) from outer (pellet) membrane. Cytoplasm and inner membrane fractions were run on SDS−PAGE and the proteins were transferred to a nitrocellulose filter using a BioRad Mini Trans-Blot™ Electrophoretic Transfer Cell according to the manufacturer's specifications. After transfer, the filter was probed with anti-TrkA antibodies, followed by several washes and probing with protein A−peroxidase conjugate. Western blots were developed with ECL Western blotting detection reagents (Amersham) and detected by exposing to X-ray film.

Protamine susceptibility assays

Protamine susceptibility assays were performed in the same minimal medium used for growth rate determinations. The medium composition was: 50 mM MES (2[*N*-morpholine]ethanesulfonic acid), 5 mM Na_2HPO_4, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 1 mM $MgSO_4$, 4 mM tricine, 10 µM $FeSO_4$. Glucose was added to a final concentration of 5 mM and glycerol and DL-lactate to final concentrations of 10 mM. The Na_2HPO_4, $(NH_4)_2SO_4$, KCl, $MgSO_4$, glucose and glycerol were autoclaved separately and added aseptically after autoclaving the buffer base. The $FeSO_4$ and DL-lactate were filter sterilized and added after autoclaving. For growth in the presence of 35 µM K^+, 20 ml fresh medium, without added K^+, was inoculated with 0.25 ml from a culture grown overnight with 0.15% glucose and 3 mM KCl. Hence, cells were grown in the presence of ~35 µM K^+, a concentration representing the amount of K^+ carried over with the inoculum. Incubation was at 37°C with rotary shaking at 200 r.p.m..

For the protamine (Calbiochem, San Diego, CA) susceptibility assays 10–20 µl log phase culture was diluted into 5 ml prewarmed growth medium to ~4×10^5 cells/ml. After 5–10 min incubation to allow mixing of the cells, a 10 µl sample was removed (the T_0 sample) and protamine added to the assay flask to a final concentration of 3 or 10 µg/ml. The protamine was prepared fresh shortly before each assay as a 1 mg/ml, MES-buffered stock solution, pH 6.3. At 30 min intervals, 10 µl samples were removed, diluted in growth medium less carbohydrate and plated in duplicate on LB plates. Data are presented as percent survival relative to the original inoculum (the T_0 sample). When testing individual plasmids for their ability to confer protamine resistance to *sapG* mutants, we streaked out individual clones onto plates containing different concentrations (0.5–1.5 mg/ml) of protamine.

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