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Salmonella typhimurium Contains an Anion-Selective Outer Membrane Porin Induced by Phosphate Starvation

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A mutant of *Salmonella typhimurium* was selected that is constitutive for the *pho* regulon. It exhibited constitutive glycerol-3-phosphate transport activity and synthesized a new outer membrane porin. Upon measurement of porin activity in black lipid films, it exhibited anion selectivity. It therefore appears analogous to the *Escherichia coli* PhoE porin.

The genetic organizations of *Escherichia coli* and *Salmonella typhimurium* are rather similar (18, 21). Known differences include the absence in *S. typhimurium* of *lac* operon genes as well as the *phoA* gene coding for the periplasmic alkaline phosphatase. Even though *phoA* is missing, the regulatory genes *phoR* and *phoB* are present (25). Recently, we described the presence of a *pho*-regulated system in *S. typhimurium*, the *ugp*-dependent transport system for *sn*-glycerol-3-phosphate (G3P) (10). Transport through this system is mediated by a periplasmic binding protein and is distinct from a *glpT*-dependent transport system for G3P that is active in membrane vesicles (10). In the present paper, we describe the isolation of a mutant that is constitutive for the *ugp*-dependent transport system. This mutant has the phenotype of a *pho* constitutive strain. It synthesizes a new outer membrane protein that exhibits properties similar to those of the PhoE porin of *E. coli*. This new protein is a murein-associated porin and forms anion-selective pores in black lipid films.

Isolation of a *pho* constitutive mutant of *S. typhimurium*. The starting strain was SH6264 (14, 15). This strain had been isolated as a mutant resistant to phages PA105 and PH51 and is therefore deficient in two outer membrane porins (the OmpD and OmpC proteins), the phage receptors. It contains as its only porin the 35-kilodalton OmpF outer membrane protein (2, 15, 22). With the help of a nearby Tn*10* insertion, we transduced *glpT*, coding for a defective *glpT*-dependent transport system for G3P, from strain RH8 (10) into strain SH6264 via P22-mediated transduction. The only remaining transport system for G3P in the resulting strain (KB66) should then be the *pho* regulon-dependent *ugp* system. In *E. coli*, G3P transported by Ugp can serve as the sole source of phosphate but not of carbon. Like all other *pho*-dependent genes, *ugp* in the wild type is expressed only under growth conditions of phosphate starvation. To select a mutant constitutive for *ugp*, we grew KB66 alternately in G plus L medium (9) with 1 mM P_i or 2 mM G3P as the only source of phosphate and with glucose as the carbon source, diluting the culture each time 100-fold. The rationale for this selection was that *ugp* constitutive mutants would not experience a lag in growth after being shifted from 1 mM P_i to 2 mM G3P. We monitored the success of the selection procedure by measuring *ugp*-dependent G3P transport after growth at 1 mM P_i. We observed the first increase of G3P transport activity after 12 growth cycles, and a 20-fold increase after 3 additional cycles. From this last culture, single cell colonies

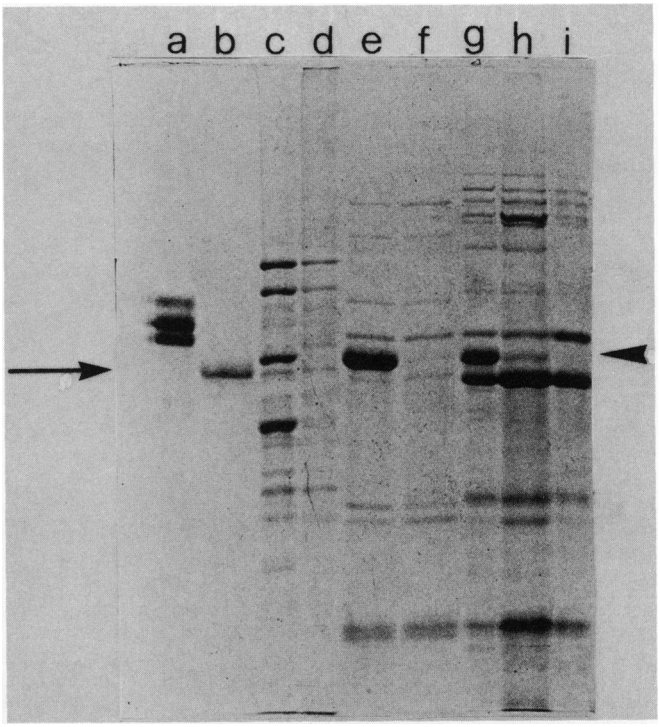

FIG. 1. Polyacrylamide gel electrophoresis of outer membrane proteins. Gels (9%) containing sodium dodecyl sulfate and 8 M urea were run according to Pugsley and Schnaitman (17). Outer membranes were isolated and separated from inner membranes as described by Hengge et al. (10). Murein-associated proteins were prepared as described by Nikaido (13). Periplasmic proteins were isolated by the cold osmotic shock procedure of Neu and Heppel (12); for *S. typhimurium*, the procedure was modified as described by Aksamit and Koshland (1). Purified PhoE porin from *E. coli* was obtained from R. Hancock, and purified OmpD, OmpF, and OmpC outer membrane proteins were obtained from T. Nakae. Lanes: a, OmpD, OmpF, and OmpC outer membrane proteins from *S. typhimurium*; b, purified PhoE porin of *E. coli*; c and d, periplasmic proteins; e and f, murein-associated proteins; g to i, outer membrane proteins; c, e, and g, *pho* constitutive strain KB17 grown in Luria broth (LB) (11); d, f, and i, *pho* wild-type strain KB66 grown in LB; h, strain grown in G plus L medium and 60 μM P_i. Each slot contained 20 μg of protein. The arrowhead indicates the position of the *pho*-dependent porin of *S. typhimurium*; the arrow indicates the position of the *E. coli* PhoE porin.

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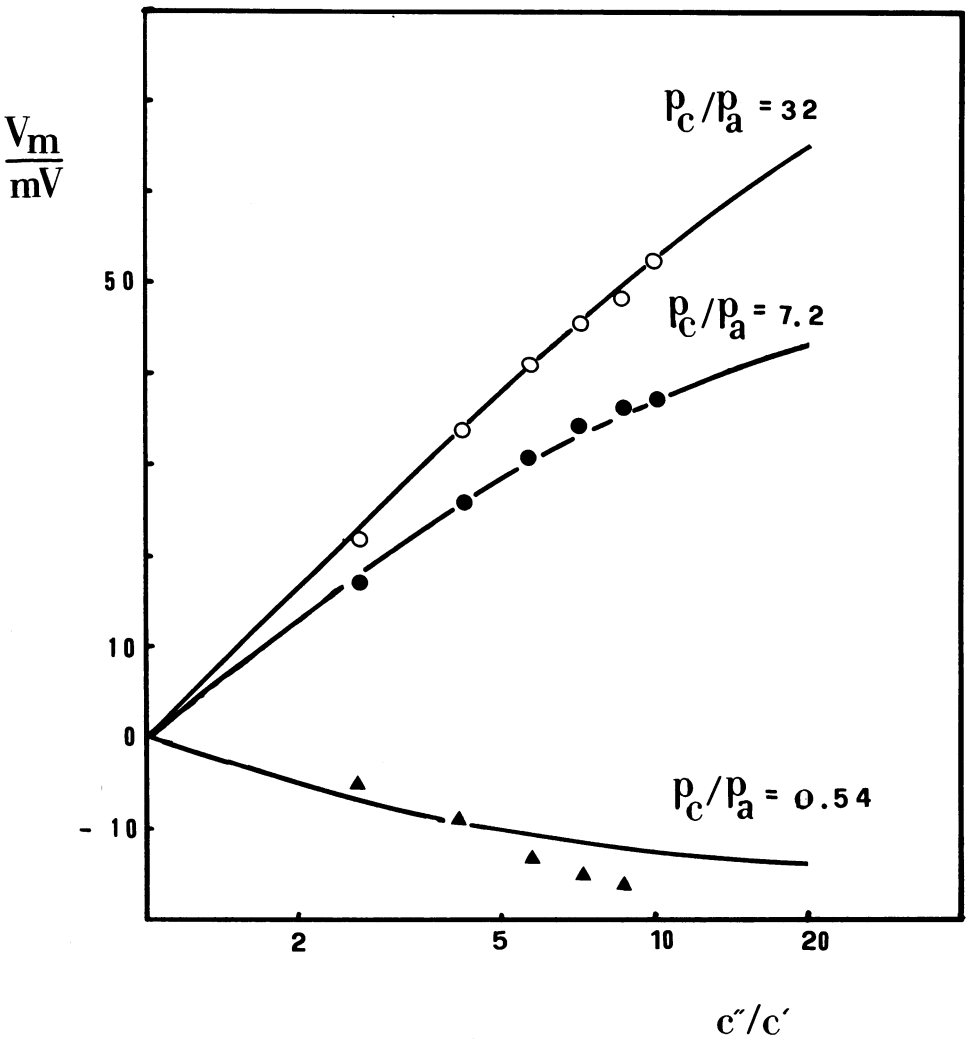

FIG. 2. Ion selectivity of *S. typhimurium* porins in black lipid films. Black lipid films made of oxidized cholesterol were used. The apparatus and methods used have been described elsewhere (6–8). Membranes were formed in a 10 mM KCl solution. After the membrane was in an optically black state, porins were added in such a concentration that a 500- to 1,000-fold increase in conductance was observed within 25 min. Then the instrument was switched to zero current potential and the salt concentration was raised by adding small amounts of 1 M KCl to one side of the membrane. The final membrane potential (V_m) was reached within 10 min. It is defined as the difference of the potential at the dilute side (c′) minus the potential at the concentrated side (c″). c′ was kept at 10 mM. The following protein preparations were used: peptidoglycan-associated proteins of the *pho* constitutive strain KB17 (▲) and of its parent strain KB66 (●). Both strains had been grown in LB at high phosphate concentrations. (○), Mixture of purified OmpC, OmpD, and OmpF *S. typhimurium* porins, the preparation shown in Fig. 1. The ion selectivity, expressed as the permeability ratio of cation to anion (P_c/P_a), was calculated by the Goldman-Hodgkin-Katz equation (6).

were isolated. Strain KB17 was chosen for further studies. At 0.25 μM external substrate concentration, it transported G3P with an initial rate of 48 pmol/min and 10^8 cells. Under the same conditions, a *pho* constitutive *E. coli* strain transported 30 pmol/min and 10^8 cells. The parent strain KB66 transported less than 0.1 and 6 pmol/min and 10^8 cells after growth at high (1 mM) and limiting P_i concentration, respectively.

The *ugp* constitutive strain contains a new outer membrane protein that is murein associated. Since the *ugp* transport system is under *pho* control (10), it was likely that the ugp constitutive strain KB17 was constitutive also for other *pho*-controlled genes. *E. coli* contains a *pho*-regulated outer membrane protein, the PhoE porin (16). We therefore analyzed membranes of the *ugp* constitutive strain KB17 by polyacrylamide gel electrophoresis, comparing the protein compositions of it and its parent strain KB66. The outer membrane of strain KB17 contains a protein band that is absent from strain KB66 grown at high phosphate concentration, but which is induced in KB66 upon phosphate starvation, though less efficiently than in KB17 (Fig. 1). This protein is murein associated and is not solubilized by heating whole cells in 2% sodium dodecyl sulfate at 60°C. Only when the temperature is raised to the boiling point or when the protein is incubated with 1% sodium dodecyl sulfate, 0.4 M NaCl, and 5 mM EDTA at 37°C (13) is its association to the murein sacculus disrupted. This property is typical for all porin proteins of *S. typhimurium* and *E. coli* (20), including the PhoE porin (16). The protein present in the outer membrane of KB17 is the most prominent murein-associated

protein in this strain, and it is not identical to the three known murein-associated porins of *S. typhimurium* (Fig. 1). In the gel system used, containing sodium dodecyl sulfate as well as urea (17), this protein exhibits an electrophoretic mobility similar, but not identical, to the mobility of the *E. coli* PhoE porin.

The gel in Fig. 1 also shows the periplasmic proteins of the constitutive strain KB17 and its parent KB66. At least two proteins absent in KB66 appear in KB17. In analogy to periplasmic proteins isolated from *pho* constitutive strains in *E. coli*, these are likely to correspond to the phosphate-binding (4) and G3P-binding proteins (3) of *E. coli*.

Therefore, it is clear that KB17 carries a mutation leading to a constitutive *pho* regulon. We have not characterized the nature of this mutation. In particular, it is not clear whether it is of the *phoR* or *phoS/T* type (23). Also, it is not clear where the gene is located that codes for the *pho*-controlled outer membrane protein in *S. typhimurium*. The *phoE* gene coding for the corresponding *E. coli* porin maps between the markers *gpt* and *proA*, at 6 min on the linkage map (5, 24). This region from *S. typhimurium* has been cloned (19). We have introduced the relevant plasmids pMR102, pSG7, and pSP1 (19) into *E. coli* CE1194, which lacks the PhoE porin but is otherwise constitutive for the *pho* regulon (24). Membranes of these plasmid-carrying strains did not synthesize the *pho*-controlled outer membrane protein of strain KB17 (data not shown). Either the structural gene for this porin does not map between *gpt* and *proA* in *S. typhimurium* or the plasmids acquired secondary mutations preventing the probably stressful overproduction of this protein in the *pho* constitutive strain CE1194 (24). Alternatively, the *phoE* gene of *S. typhimurium* may not be correctly regulated in *E. coli* (Tommassen, personal communication).

The *pho*-controlled outer membrane protein forms anion-selective pores in black lipid films. When incorporated into black lipid membranes, porins form water-filled channels that increase the ion conductivity across the membrane (7, 8). By applying different concentrations of the same salt on opposite sides of the membrane, one obtains a zero current potential across the membrane if the pores exhibit a selectivity for cations or anions. The major porins of *E. coli*, OmpF and OmpC, as well as the major porins of *S. typhimurium*, the OmpD, OmpF, and OmpC proteins, exhibit cation selectivity (7; R. Benz, A. Schmid, and R. E. W. Hancock, submitted for publication), whereas the *E. coli* PhoE pore exhibits anion selectivity (6), consistent with the view that this protein participates in the permeation of phosphates through the outer membrane. Consequently, we used the murein-associated porins of the *pho* constitutive strain KB17 as well as its parent KB66 and tested their ion selectivities in black lipid films. Both preparations still contained the cation-selective OmpF porin, whereas the preparation of strain KB17 contained, in addition and as the major component, the *pho*-controlled porin (Fig. 1). The amount of protein used in the assay was adjusted so that the black lipid membrane incorporated ca. 100 channels in each experiment.

The preparation of the parent strain KB66 exhibited cation selectivity; the preparation of the *pho* constitutive strain exhibited anion selectivity (Fig. 2).

In conclusion, we isolated an *S. typhimurium* strain, KB17, that carries a mutation leading to a constitutive *pho* regulon. The consequences of this mutation include the appearance of a new anion-selective outer membrane protein, a transport system for G3P, and two new periplasmic proteins analogous to *pho* regulon products in *E. coli*.

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