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Physical Mapping of the K^+ Transport *trkA* Gene of *Escherichia coli* and Overproduction of the TrkA Protein

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The position on the *Escherichia coli* chromosome of *trkA*, a gene coding for a membrane protein involved in K^+ transport by the constitutive uptake system Trk, was determined. We observed that the gene is transcribed in a clockwise direction and that it is located at 72.4 min on the chromosome in a 1.75-kilobase *Nru*I-*Eco*RV DNA fragment 1.0 kilobase upstream of *rplQ*. We localized an additional gene encoding a 17,000-molecular-weight protein of unknown function between the *trkA* and *rplQ* genes. A plasmid, pDB3, was constructed in which the transcription of the *trkA* gene was put under the control of the λ p_L promoter. pDB3-containing cells of a strain, which contained the temperature-sensitive λ repressor *c*I857 in the chromosome, overproduced the 53,000-molecular-weight TrkA protein at the nonpermissive temperature to such an extent that TrkA became the major cell protein. From cell fractionation studies, we conclude that the overproduced TrkA protein forms aggregates.

Bacterial cells accumulate K^+ ions to high concentrations in the cytoplasm. This high K^+ content plays a role in the maintenance of cell turgor pressure, in the activation of enzymes, and possibly in cytoplasmic pH homeostasis (for reviews, see references 5, 12, 14, 17, and 18). The high internal K^+ concentration is maintained by the balance of activities of K^+ uptake and K^+ exit systems. *Escherichia coli* is able to express two K^+ uptake systems (33). The inducible system Kdp functions as a K^+-translocating ATPase and has been characterized in detail (for a review, see reference 11). Much less is known about the second K^+ uptake system, Trk, which is expressed constitutively. Trk transports K^+ with a 10^3-fold lower affinity but severalfold larger capacity than Kdp (33) does and is therefore responsible for K^+ uptake under most conditions of growth. The activity of the Trk system is regulated by the cell turgor pressure (25, 32), and Trk also requires both a high cytoplasmic ATP concentration and a high transmembrane proton motive force for activity (31). It has been proposed that ATP, or another as yet unidentified metabolite, activates the system, whereas the proton motive force yields the driving force for K^+ uptake (4, 36, 37). Trk transport activity is diminished by mutations in *trkA*, *trkD*, *trkE*, *trkG*, and *trkH* dispersed on the chromosome (33; D. C. Dosch, Ph.D. thesis, University of Chicago, Chicago, Ill., 1985). This suggests that the Trk system is composed of different polypeptides, but nothing is known about its assembly and polypeptide composition in the membrane. These properties make Trk a unique type of bacterial transport system, separate from both the group of transport ATPases and from that of simple secondary porters (15, 16, 36). Mutations in the *trkA* gene greatly reduce the rate of K^+ transport, and the TrkA protein might be the subunit that binds K^+ (G. C. Helmer and W. Epstein, personal communication). Starting with a plasmid that contains the *trkA* gene on an 8-kilobase (kb) *E. coli* chromosomal fragment (pGH1; G. C. Helmer and W. Epstein, Biophys. J. 33:61A, 1981), we subcloned the *trkA* gene and determined its position and direction of transcription on the chromosome. In addition, we describe the overproduction of the TrkA protein, when under the control of the λ p_L promoter, to become the major protein in the cell.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains were all derived from *E. coli* K-12. Strains TK1001 (F^- *thi lacZ gal rha kdpABC5 trkD1*) (33), TK2205 (F^- *thi lacZ rha nagA kdpABC5 trkA405 trkD1*), and TK2105-*recA* (TK2205 *malA*) were obtained from W. Epstein, University of Chicago, Chicago, Ill. Strain DS410 (*lacY rpsL xyl mtl minA minB thi*) (29) was obtained from K. Hantke, University of Tübingen, Tübingen, Federal Republic of Germany, and strain ΔH1 Δ*trp* [Str^r *lacZ*(Am) Δ*bio-uvrB* Δ*trpEA2* (λ *N*am7-*N*am53 *c*I857 ΔH1)] (30) was obtained from J. Lengeler, University of Osnabrück, Osnabrück, Federal Republic of Germany.

Plasmid pGH1 was obtained from W. Epstein. Plasmid pPLa2311 (30; see Fig. 4) was obtained from E. Remaut, via L. Wieczorek, University of Osnabrück. Plasmids pAH100 (Tc^r), pAH101 (Ap^r), pAH106 (Ap^r), and pAH108 (Ap^r) were constructed by insertion of DNA restriction fragments of pGH1 or pAH100 into suitable sites of plasmid pBR322 (Fig. 1). Plasmids pAH102, pAH103, and pAH104 (all Tc^r) were generated by cleavage of pAH100 at its *Pvu*I site (Fig. 1C), partial digestion of the DNA by exonuclease III and S1 nuclease action (34), and intramolecular ligation of the DNA fragments obtained. Plasmid pDB3 (Km^r) was constructed by insertion of the 3.0-kb *Pst*I insert of pAH100 into the *Pst*I site of plasmid pPLa2311 (see Fig. 4).

Growth conditions. Cells were grown on the rich medium KML (1% tryptone, 0.5% yeast extract, 1% KCl [all wt/vol]) or on minimal medium containing various K^+ concentrations, with glucose (10 mM) as the carbon source (14). If necessary, thiamine (1 μg/ml), kanamycin (25 μg/ml), tetracycline (15 μg/ml), carbenicillin (100 μg/ml), or D-cycloserine (20 μg/ml) was added at the final concentrations indicated.

Recombinant DNA techniques. Plasmid preparations, DNA restrictions, DNA ligations, and transformations of cells

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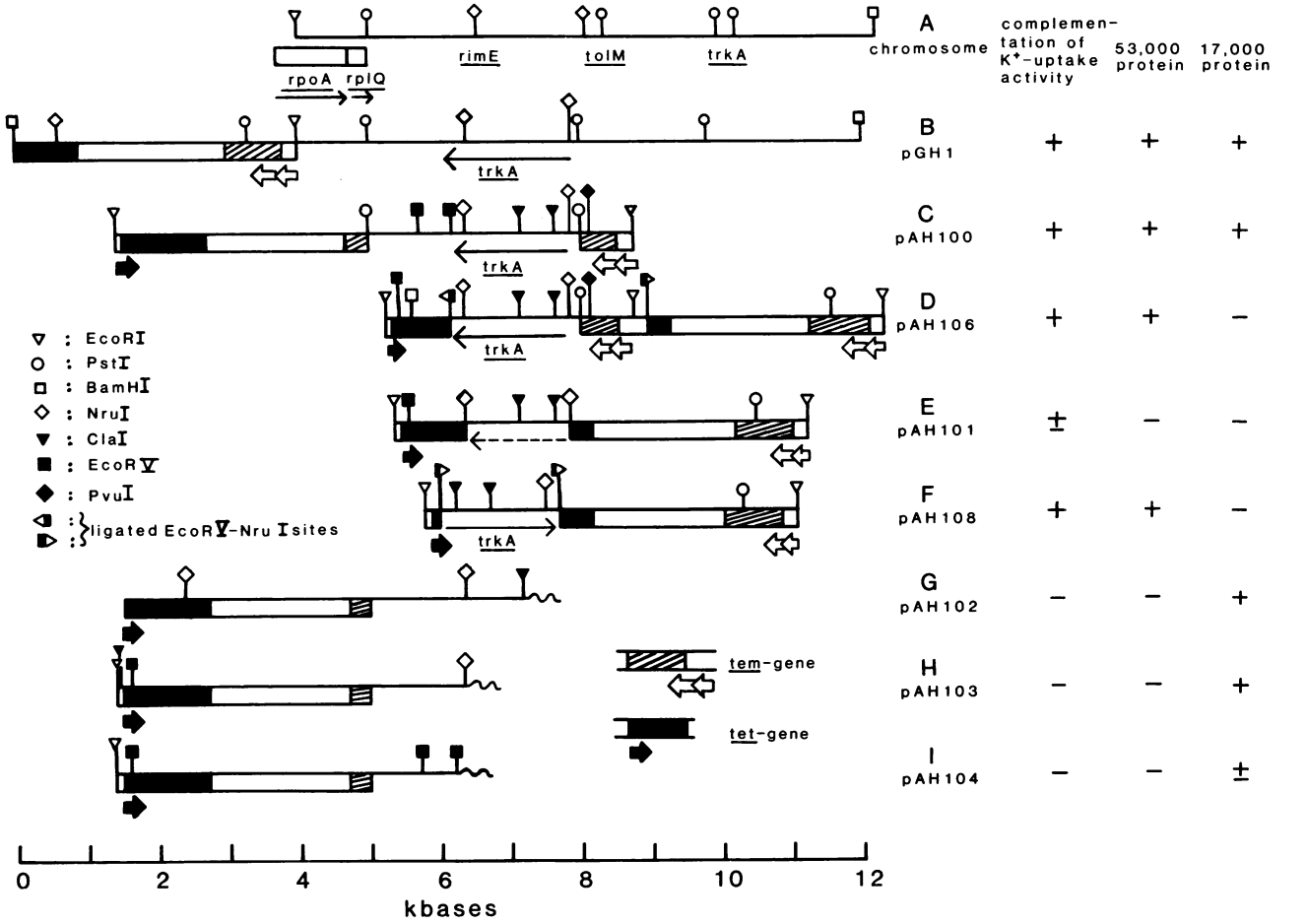

FIG. 1. Plasmids used in the physical mapping of the *trkA* gene. (A) Part of the 72.4-min area of the chromosome (24); the clockwise direction is from right to left. The position and the direction of transcription of the sequenced *rpoA* and *rplQ* genes are indicated. The approximate position of the *rimE*, *tolM*, and *trkA* genes in panel A are taken from reference 24. (B to I) Plasmids used to determine the position and direction of transcription of the *trkA* gene, indicated by the thin arrow under the plasmids in panels B, C, D, and F. The truncated *trkA* gene in pAH101 is indicated by the dashed arrow (panel E). Vector DNA is indicated by double lines, and chromosomal DNA inserts are indicated by single lines. Note that the orientation of the insert in pAH108 is opposite to that in the other plasmids. The *tem* and *tet* genes originating from the vector pBR322 are shown as hatched and solid areas, and their promoters are shown as open and solid large arrows, respectively. The right-handed side of the figure summarizes the properties of the plasmids.

with plasmids were carried out by established procedures (23).

Complementation analysis. Cells from single colonies of strains TK2205 or TK2105-*recA* containing plasmids to be tested for their TrkA complementation activity were patched on plates with minimal growth medium supplemented with 0.3, 1, 3, 10, 30, or 115 mM K^+. Plasmids conferring full complementation activity enabled the cells to grow at K^+ concentrations of 0.3 mM and above. Plasmid pBR322 (run as a control) enables cells to grow at 10 mM K^+ and above (8).

Plasmid-encoded protein synthesis by minicells. Plasmid-containing cells of strain DS410 were grown overnight, and minicells were isolated on a sucrose gradient (29). Most of the labeling experiments were carried out with fresh cells, but it was later observed that results obtained with cells stored frozen at −40°C were as good as those obtained with fresh cells. Labeling with [^{35}S]methionine was done with 4 ml of a suspension of minicells diluted to an optical density of 0.5 (approximately 5×10^9 minicells per ml) in medium K115 (13), in which the sulfate was replaced by an equimolar equivalent of chloride. This suspension was first preincubated with 20 mM glucose and 20 μg of D-cycloserine per ml for 30 min at 37°C with shaking. To this suspension were then added 5 μl of Difco methionine assay medium (dissolved as specified by the supplier [Difco Laboratories, Detroit, Mich.]) and 20 μCi of [^{35}S]methionine. The cells were incubated under the same conditions for 1 h and then centrifuged for 10 min at 16,500 × *g*. The cell pellet was denatured by treatment with 5% trichloroacetic acid. The precipitated proteins were sedimented by centrifugation, washed twice with buffer containing 10 mM Tris-acetate, 10 mM $MgCl_2$, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.8), and dissolved in 100 μl of buffer containing 50 mM Tris hydrochloride, 10% mercaptoethanol, 2% sodium dodecyl sulfate, and 12.5% glycerol (final pH 6.8) (sample buffer). After this suspension had been boiled for 5 min, the proteins were separated by electrophoresis on a gel containing a linear gradient of 7.5 to 17.5% polyacrylamide prepared by the method of Laemmli (20). Dried gels were overlaid with

Kodak XAR-5 X-ray film (Eastman Kodak Co., Rochester, N.Y.) for autoradiography and exposed for several days.

Overproduction of the TrkA protein. Cells of strain ΔH1 Δ*trp* containing plasmid pDB3 were grown at 28°C in medium KML in the presence of 25 μg of kanamycin per ml. After the optical density of the suspension had reached a value of 0.4 at 578 nm, the temperature of the suspension was shifted to 42°C, and the optical density and the protein pattern of the cell suspension were measured as a function of time.

Fractionation of cells overproducing the TrkA protein. Cells of strain ΔH1 Δ*trp*, growing in five batch cultures of 1 liter each, reached a maximal optical density of 0.82 at 70 min after the temperature shift to 43°C. After another 20 min (during which the optical density had dropped to 0.65), the culture flasks were cooled in ice-water for 10 min. Subsequently, the cells were harvested by centrifugation, and the cell pellets were combined, suspended in 50 ml of buffer (pH 8) containing 50 mM sodium 3-(*N*-morpholino)propanesulfonic acid, 10 mM $MgCl_2$, 175 mM KCl, 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride (buffer 1) and stored frozen at −80°C. For use, the cells were rapidly thawed, washed once by centrifugation with 50 ml of the same buffer, resuspended at the same cell concentration, and subjected to Ribi-press (Ribi cell fractionator RF-1; Ivan Sorvall, Inc., Norwalk, Conn.) treatment at a pressure of 138 MPa at 5 to 10°C. The suspension was then centrifuged for 10 min at 1,500 × *g*. The pellet (fraction P1) was taken up in 1.25 ml of buffer 1 and stored frozen at −30°C. The supernatant was centrifuged for 20 min at 21,000 × *g*. The pellet (fraction P2) was taken up in 12.5 ml of buffer 1 and stored frozen. The supernatant was then centrifuged for 90 min at 250,000 × *g*, yielding pellet fraction P3, which was suspended in 3.2 ml of buffer 1 and subsequently frozen. The supernatant containing soluble cellular proteins was frozen as such. Control cells grown at 28°C were harvested at an optical density of 0.92, and subsequent steps were as for cells that were derepressed for overproduction. The protein content of the different fractions was determined. Portions of these fractions were suspended in sample buffer such that the protein content was 1 mg/ml. The samples were boiled for 5 min, and 20 μg of protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide electrophoresis on a gel formed by the method of Lugtenberg et al. (22).

The inner and outer membranes of cells that had overproduced the TrkA protein were separated on a sucrose step gradient by the method described by Osborn and Munson (27), except that lysis was accomplished by sonication of 20-ml aliquots of the suspension for three periods of 25 s (50% duty cycle) transferred by the big tip of a Branson B15 sonifier (Branson Sonic Power Co., Danbury, Conn.).

Other methods. Cells were depleted of K^+ by treatment with Tris and EDTA. The K^+ uptake activity of these cells was determined at pH 7.5 by flame photometry (3). Peptide mapping of the TrkA protein with V8 protease was carried out as described by Cleveland et al. (7). Protein was determined by a modification of the method of Lowry et al. (21), as described in reference 9.

Chemicals. [^{35}S]methionine (800 Ci/mmol) was from Dupont/New England Nuclear, Dreieich, Federal Republic of Germany. Restriction enzymes, T4 DNA ligase, exonuclease III, S1 nuclease, alkaline phosphatase, and CsCl were either from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or from Boehringer GmbH, Mannheim, Federal Republic of Germany. V8 protease and other special compounds were from Sigma Chemie GmbH, Deisenhofen, Federal Republic of Germany.

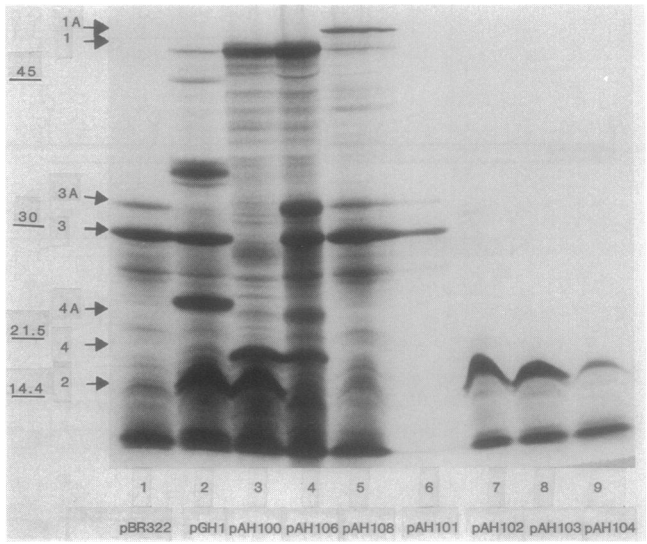

FIG. 2. Plasmid-encoded proteins expressed in minicells. Cells of strain DS410 containing the plasmids indicated under the different lanes were labeled as described in Materials and Methods. The numbered bands indicate the following proteins: 1, TrkA protein; 1A, fusion protein between the amino-terminal end of the *tet* gene product and the TrkA protein; 2, unknown MW 17,000 protein; 3, β-lactamase; 3A, pre-β-lactamase; 4, truncated β-lactamase; 4A, precursor of the truncated β-lactamase. The MW of the marker proteins (10^3) are given on the left-hand side of the gel.

RESULTS

Subcloning of the *trkA* gene. A restriction map has been published of the 72.4-min area of the *E. coli* chromosome (24), where the *trkA* gene is located (1, 13). Plasmid pGH1 carries the *trkA*-containing chromosomal fragment inserted between the *Eco*RI and *Bam*HI sites of plasmid pBR322 (Fig. 1B). The restriction map of the insert of pGH1 was closely similar to that reported by Meek and Hayward (24) (Fig. 1A). These authors showed that the left-hand end of the DNA fragment shown in Fig. 1A carries part of the *rpoA* gene and that the *rplQ* gene continues 10 bases beyond the left-hand *Pst*I site. We therefore expected the *trkA* gene to be located between this *Pst*I site and the *Bam*HI site of the chromosomal insert of plasmid pGH1. We cloned the different *Pst*I and *Bam*HI-*Pst*I restriction fragments of pGH1 into suitable plasmid vectors, transformed cells of strain TK2205 (*kdp trkA trkD*) with these plasmids, and checked for complementation of K^+ transport activity as indicated by growth on plates containing 0.3 mM K^+. The 3.0-kb *Pst*I-*Pst*I fragment containing the two *Nru*I sites (Fig. 1B), cloned into the *Pst*I site of pBR322 (plasmid pAH100; Fig. 1C), showed the desired activity.

Plasmid-encoded proteins. Figure 2, lanes 1 to 3, shows the proteins expressed in a minicell system by plasmids pBR322, pGH1, and pAH100, respectively. Although pGH1 (lane 2) coded for polypeptides with molecular weights (MW) 53,000 (band 1 in Fig. 2; identified as the TrkA protein [Helmer and Epstein, personal communication]), 48,000, 36,000, 30,000 (vector-encoded β-lactamase; band 3 in Fig. 2), 24,000, and 17,000 (band 2), the shorter pAH100 (lane 3) expressed only

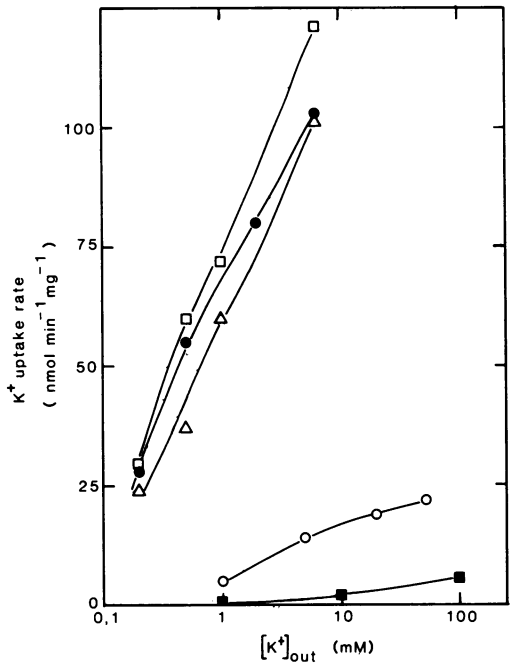

FIG. 3. K^+ uptake by plasmid-containing cells. Tris–EDTA-treated cells were suspended at 1 mg (dry weight) per ml of 200 mM sodium 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate medium of pH 7.5. The suspension was shaken for 10 min at 20°C in the presence of 10 mM glucose. Then K^+ was added to the concentration indicated. The initial rate of K^+ uptake was calculated from the K^+ contents of pellets from 1.0-ml samples centrifuged at different times through silicone oil. Symbols for strain TK2105-*recA* containing one of the following plasmids: (○) pAH101; (●) pAH100; (□) pAH108; (△) pAH106; (■) pBR322. The kinetic parameters for K^+ uptake were as follows: K_m, 0.4, 0.65, 0.65 and 5 mM; and V_{max}, 125, 110, 150 and 25 nmol min^{-1} mg^{-1} for the strains containing plasmids pAH100, pAH106, pAH108, and pAH101, respectively. Wild-type TrkA activity in strain TK1001 shows under similar conditions a K_m of 0.5 mM and a V_{max} of 180 nmol min^{-1} mg^{-1} (3).

the proteins with MW 53,000 and 17,000 (Fig. 2, lane 3), as well as an additional protein with MW 20,000 (band 4 in Fig. 2). The latter probably represents a truncated form of the β-lactamase, which can occur upon cloning into the *Pst*I-site of pBR322 (19, 26).

Deletion experiments. The chromosomal insert of pAH100 contained two restriction sites for the enzymes *Eco*RV, *Cla*I, and *Nru*I but none for *Ava*I, *Sal*I, *Pvu*II, *Sca*I, or *Sph*I (Fig. 1B).

The shortest DNA fragment of pAH100 with full complementation activity was the 1.75-kb *Eco*RV-*Nru*I fragment in pAH108 (Fig. 2). This plasmid still encoded the TrkA protein, but not that with MW 17,000 (Fig. 2, lane 5). However, pAH108 also encoded an additional MW 58,000 protein. We assume that this represents a fusion protein between the amino-terminal end of the *tet* gene product and the complete TrkA protein, which occurred as a result of the cloning of the insert into the *Eco*RV site of plasmid pBR322, located in the coding region of the *tet* gene (Fig. 1F). Plasmid pAH106, which contained the slightly larger 2.0-kb *Eco*RV-*Pst*I insert (Fig. 1D), encoded the MW 53,000 protein (Fig. 2, lane 4), as well as both the complete and the truncated β-lactamases and their precursors (bands 3, 4, 3A, and 4A in Fig. 2, respectively).

Some complementation of K^+ transport activity was observed for the 1.5-kb *Nru*I-*Nru*I fragment from the insert of pAH100 in pAH101 (Fig. 1E). pAH101-containing minicells, however, produced neither the MW 53,000 nor a shortened TrkA protein, but only the complete β-lactamase and its precursor (Fig. 2, lane 6, and experiments not shown, in which we overloaded the gel with protein expressed by pAH101-containing minicells). The complementation activity of pAH101-containing cells was due to a 10-fold stimulated rate of K^+ uptake compared with that of pBR322-containing cells (Fig. 3), indicating that the partial complementation activity induced by pAH101 is not an artifact due to K^+ uptake by the tetracycline exit system encoded by the *tet* gene (8). The K^+ uptake activity of pAH101-containing cells was, however, still much lower than that expected for wild-type Trk activity. This was due to both an increased K_m and a decreased V_{max} of the K^+ uptake system of the pAH101-containing cells (Fig. 3).

From these results, we conclude that the *trkA* gene is located on the 1.75-kb *Nru*I-*Eco*RV DNA fragment as indicated in Fig. 1 and that the direction of transcription of the gene in pGH1 and pAH100 is from right to left, which corresponds to a clockwise direction of transcription on the chromosome.

We also established that the gene coding for the MW 17,000 protein is located between the *trkA* and *rplQ* genes (cf. Fig. 1), since DNA fragments which were generated by digestion with exonuclease III from the *Pvu*I site in pAH100 (Fig. 1B) and which contained the left-hand *Cla*I site of the insert of pAH100 (pAH102; Fig. 1G), the left-hand *Nru*I site (pAH103; Fig. 1H), or the *Eco*RV sites (pAH104; Fig. 1I) did not complement for K^+ transport activity, but all caused expression of the MW 17,000 protein in the minicell system (Fig. 2, lanes 7 to 9). This gene of unknown function was not *tolM*, which is placed with an uncertainty asterisk in this part of the chromosome map (1) (Fig. 1A), since plasmid pAH100 did not complement for TolM activity (K. Hantke, unpublished observations).

Overproduction of the TrkA protein. The intensity of the TrkA protein band expressed in the minicell system varied from plasmid to plasmid (Fig. 2). However, if the intensity of this band is compared with that of either the complete or the truncated β-lactamase, it is seen that both the promoters of the *tem* and *tet* genes cause enhanced expression of the

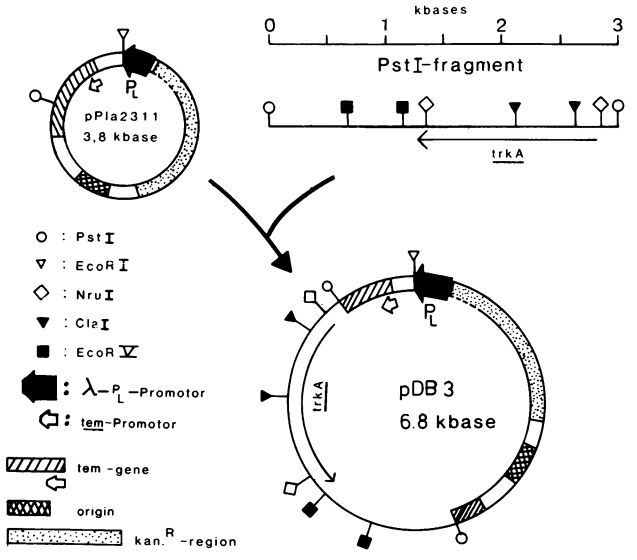

FIG. 4. Construction of plasmid pDB3. For details, see the text.

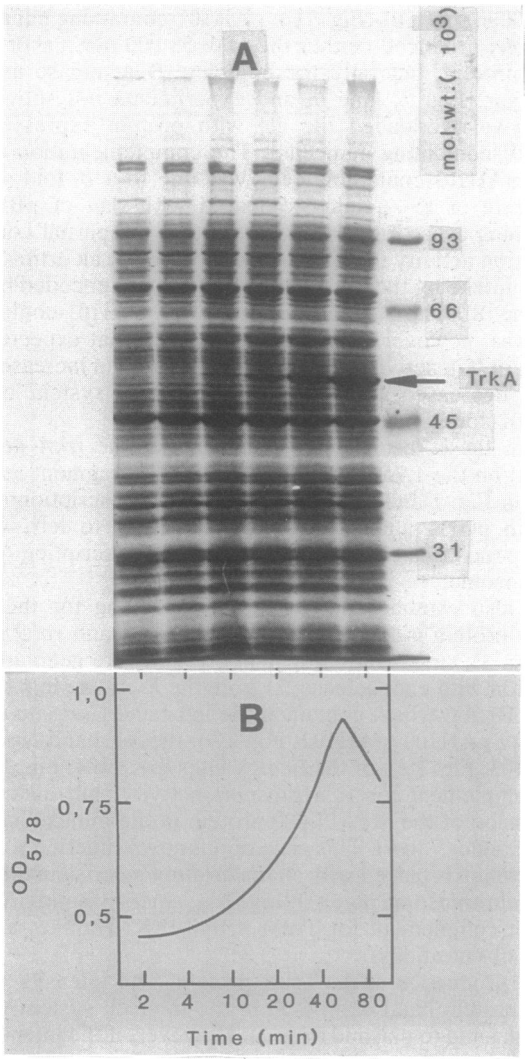

FIG. 5. Overproduction of the TrkA protein. Cells of strain ΔH1 Δ*trp*(pDB3) were grown as described in Materials and Methods. The pattern of total cell proteins (A) and the optical density (OD_{578}) of the suspension (B) were measured as a function of time after the temperature shift to 42°C at zero time. The arrow indicates the position of the TrkA protein. The optical density of the suspension reached a maximum of 0.93 at 60 min after the shift and decreased to 0.78 at 100 min.

TrkA protein (plasmids pAH100, pAH106 and pAH108, respectively; Fig. 1 and Fig. 2, lanes 3, 4, and 5). This overproduction was insufficient for isolation of the protein, since Coomassie-blue stained TrkA protein was detected only in gels run with total proteins from plasmid pAH100-containing minicells but not in gels of total proteins from normal, pAH100-containing cells (not shown).

A larger overproduction of the TrkA protein was achieved by using the expression system developed by Remaut et al. (30). Their plasmid pPLa2311 (Fig. 4) contains (i) the λ p_L promoter directing transcription in a counterclockwise direction, and (ii) 300 bases away from this promoter, the *tem*-β-lactamase gene from pBR322 in the same orientation. We inserted the *trkA* gene containing the 3.0-kb *Pst*I fragment, which was previously used to generate pAH100 (Fig. 1B and C), into the *Pst*I site inside the *tem* gene of the vector, generating plasmid pDB3 (Fig. 4). We transformed cells of strain ΔH1 Δ*trp*, which contains the gene encoding the temperature-sensitive λ repressor *c*I857 in the chromosome (30), with this plasmid and selected for transformation by growth on kanamycin plates (Fig. 4). The transformants were tested for the presence of the *Pst*I insert by lack of growth on carbenicillin and for enhanced transcription by growth inhibition, induced by a temperature shift, from 28 to 42°C, of cells growing in liquid medium. Cells with this phenotype contained plasmid pDB3 (Fig. 4). These cells produced a polypeptide with MW 53,000 as the major cell protein after the shift to 42°C (Fig. 5). Limited proteolysis with V8 protease indicated that this polypeptide is identical to the TrkA protein produced by minicells containing plasmid pAH100 (Fig. 6).

Cell fractionation. Experiments with Triton X-100 treatment of total membranes derived from TrkA-expressing maxicells suggest that the protein is located in the cytoplasmic membrane (Helmer and Epstein, personal communication). To determine whether this was also the case for the overproduced TrkA protein, cells were subjected to Ribipress treatment and fractionated by differential centrifugation. The protein pattern of these fractions was analyzed by gel electrophoresis (Fig. 7). Amazingly, the overproduced TrkA protein accumulated in the pellet fractions P1 and P2 (Fig. 7, lanes 4 and 6). Moreover, the yield of these fractions was considerably higher than that of those derived from control cells grown at 28°C (legend to Fig. 7). The pellet obtained after ultracentifugation (fraction P3; mainly cytoplasmic membranes) contained much less TrkA protein (lane 8), and the soluble protein fraction was almost free of it (lane 10).

Even after pretreatment with EDTA plus EGTA (2 mM each), we were unable to dissolve the overproduced TrkA protein by treatment with cholate, deoxycholate, or a number of neutral detergents, indicating that the overproduced protein might occur in aggregates. This notion is supported

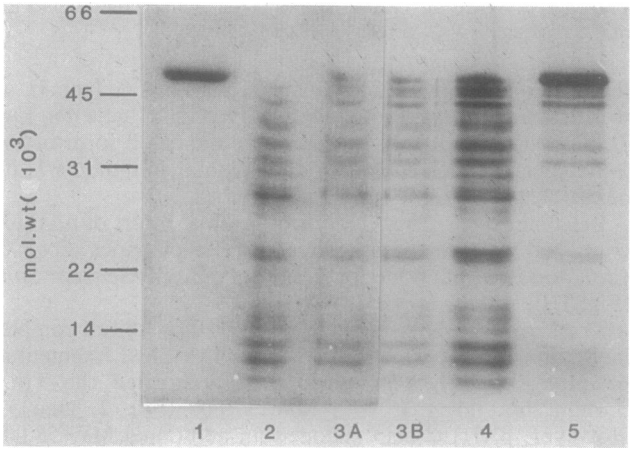

FIG. 6. Protein mapping of the TrkA protein with V8 protease. The overproduced MW 53,000 protein from strain ΔH1 Δ*trp*(pDB3) or the ^{35}S-labeled TrkA protein from minicells of strain DS410(pAH100) were cut out from gels, partially digested with V8 protease, and analyzed for the size of the generated fragments by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7). Lanes 1 to 3A show Coomassie blue-stained polypeptides, and lanes 3B to 5 show autoradiograms of ^{35}S-labeled polypeptides. Lanes: 1, MW 53,000 protein produced by strain ΔH1 Δ*trp*(pDB3); 2, the same protein digested with V8 protease; 3A and 3B, the same protein mixed with the radioactively labeled TrkA protein, subsequently digested with the protease; 4, digested radioactive TrkA protein; 5, radioactive TrkA protein.

by the result of an experiment in which we separated inner and outer membranes of TrkA-overproducing cells on a sucrose gradient by the method described in reference 27. The overproducing cells were filamentous. The separation of the membranes (Fig. 8) was, nevertheless, closely similar to that of control cells (not shown) and resembled the pattern reported in the literature (27), since the outer membrane proteins A and C/F were confined mainly to fractions H and M, as indicated by the protein pattern in the region where proteins with MW 33,000 to 38,000 are located (Fig. 8, lanes 2 and 3). In addition to the H, M, L2, and L1 bands (27; Fig. 8, lanes 2 to 5), overproducing cells yielded an additional, even heavier band, which sedimented on top of the layer containing 55% sucrose. This band contained mainly TrkA and was essentially free of the outer membrane proteins A and C/F (Fig. 8, lane 1).

We recently succeeded in dissolving the TrkA protein with the detergent tetradecyltrimethylammonium bromide.

DISCUSSION

We showed in this work that a 3.0-kb *Pst*I DNA fragment, located in the counterclockwise direction next to the *rplQ* gene at 72.4 min on the *E. coli* chromosome (Fig. 1) (24), encodes two proteins with MW 17,000 and 53,000 (Fig. 3). We confirmed the conclusion of Helmer and Epstein (personal communication) that the MW 53,000 protein is the *trkA* gene product, since we observed that plasmids that complemented for K^+ transport activity encoded the protein in a minicell system, whereas plasmids without or with only some complementation activity did not encode this polypeptide (Fig. 1 to 3).

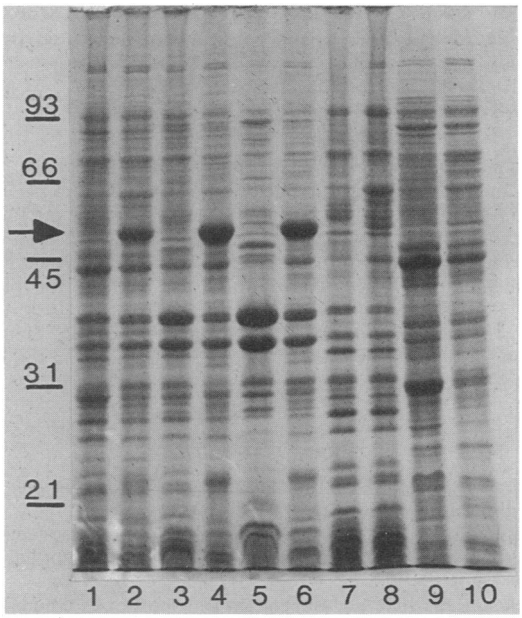

FIG. 7. Fractionation of TrkA protein-overproducing cells. Even-numbered lanes show the protein pattern of fractions derived from cells of strain ΔH1 Δ*trp*(pDB3) derepressed for TrkA protein synthesis by growth at 42°C, and odd-numbered lanes show the same fractions of cells grown at 28°C, at which TrkA synthesis was repressed. Lanes: 1 and 2, intact cells; 3 and 4, fraction P1 (mainly intact cells); 5 and 6, fraction P2 (mainly large membrane fragments); 7 and 8, fraction P3 (small membrane vesicles); 9 and 10, soluble proteins. The total protein content of the different fractions was 374, 27, 116, 63, and 94 mg for cells, fractions P1, P2, and P3, and soluble proteins from cells shifted to 43°C, respectively, and 390, 9, 25, 88, and 148 mg for the same fractions from cells grown at 28°C. The arrow indicates the position of the TrkA protein. The MW of the marker proteins (10^3) is given on the left-hand side of the gel.

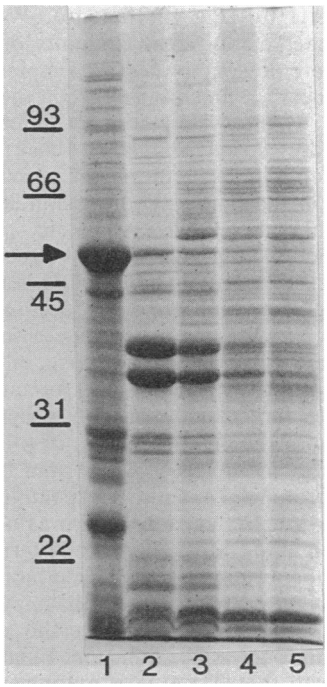

FIG. 8. Membrane separation of cells that had overproduced the TrkA protein. Lanes: 1, material that sedimented on the cushion of 55% (wt/wt) sucrose; lanes 2 to 5, fractions H, M, L2, and L1, respectively (27). The arrow indicates the position of the TrkA protein. Each lane contained about 10 to 20 μg of protein. The yield (in milligrams of protein) of the fraction of lane 1 was about the same as that of the H fraction (lane 2). The MW of the marker proteins (10^3) is given on the left-hand side of the gel. The arrow indicates the position of the TrkA protein.

Further subcloning showed that the *trkA* gene is located on a 1.75-kb *Eco*RV-*Nru*I DNA fragment (Fig. 1 to 3). This means that if the MW of the TrkA protein (53,000) is indicated correctly by the gel system (Fig. 2), this DNA fragment cannot contain much genetic information apart from that required for the expression of the *trkA* gene. DNA-sequencing experiments, which are in progress, will show whether this is indeed the situation.

The direction of transcription and translation of the *trkA* gene is clockwise on the *E. coli* chromosome. This conclusion is based (i) on the known orientation and location of the cluster of ribosomal genes adjacent to the DNA fragments analyzed (Fig. 1A) (24), (ii) on the orientation of the *trkA* gene in plasmids in which strong promoters directed the overproduction of the protein (Fig. 1, 2, 4, and 5), (iii) on the fact that plasmids with deletions in the *trkA* gene distal to the ribosomal genes did not cause partial complementation of transport activity (Fig. 1G to I), and (iv) on the observation that the deletion of a 0.4-kb *Nru*I-*Eco*RV fragment proximal to the ribosomal genes led to some complementation (plasmid pAH101; Fig. 1E and 3).

Plasmid pAH101 probably encodes a truncated TrkA protein that lacks at most 130 amino acids at the carboxy-terminal end as a result of the deletion created in vitro. In contrast to the results with a strain bearing the amber *trkA457* mutation (Helmer and Epstein, personal communication), we were not able to visualize the truncated protein

encoded by pAH101 (Fig. 2, lane 6). We nevertheless assume that undetectably small amounts of the truncated TrkA protein were present in the Trk complex, causing both a reduction in the V_{max} and an increase in the K_m values for transport of K^+ by the system (Fig. 3). Interestingly, these characteristics are closely similar to those of some TrkA mutants (Helmer and Epstein, personal communication), suggesting that in these strains, the lesion is located in the carboxy-terminal end of the TrkA protein.

The complementing plasmid pAH108 directed the expression of both the TrkA protein and an additional MW 58,000 polypeptide (Fig. 2, lane 5). We consider it unlikely that this MW 58,000 protein represents a precursor form of the MW 53,000 TrkA protein. If this were the case, one would expect that in Fig. 2, lane 4 (pAH106), this precursor would be observed in even larger amounts than in lane 5 (pAH108), since processing of the precursor form of β-lactamase to its mature form is less complete in the former lane than in the latter (Fig. 2, proteins 3A and 3, respectively). However, neither in lane 4 nor in lanes 2 and 3 was any MW 58,000 protein observed at all. Moreover, the N-terminal sequence of the MW 53,000 protein starts with methionine (D. Bossemeyer, E. P. Bakker, and I. R. Booth, unpublished observations), which might argue against the existence of a precursor with an N-terminal extension. Finally, the existing evidence suggests that TrkA is a cytoplasmic membrane protein (Helmer and Epstein, personal communication), and very few bacterial cytoplasmic membrane proteins are known to possess a precursor sequence.

We propose that the MW 58,000 protein represents a fusion between the N-terminal end of the Tet protein and the TrkA protein. A total of 99 bases are located between the start of the coding region of the *tet* gene and the *Eco*RV site, where the chromosomal fragment of pAH108 starts (2, 28). If the *trkA* gene starts not too far beyond this ligated *Eco*RV-*Nru*I site (Fig. 1F), and if the reading frame of the two genes in the recombinant is the same, an increase of the MW of the protein by about 5,000 is possible. A final condition for the expression of the fusion protein is that in pAH108, between the start of the coding regions of the *tet* and *trkA* genes, no strong signals exist that direct the termination of translation. The fact, then, that pAH108-containing minicells also synthesized the MW 53,000 TrkA protein indicates that the chromosomal insert of this plasmid still carries the information required for the separate expression of the TrkA protein.

Our results support the notion that the presence of the TrkA protein alone is not sufficient for K^+ uptake activity (14): cells that overproduced the TrkA protein showed similar transport activities (Fig. 3) to those of haploid $TrkA^+$ cells (3). This lack of increased activity was not due to a completely erroneous subcellular localization of the overproduced TrkA protein, since our data indicate that under conditions at which the formation of TrkA aggregates was observed (Fig. 7 and 8), the TrkA protein was also present in the membrane fractions that consisted mainly of cytoplasmic membranes (Fig. 7, lane 8; Fig. 8, lane 5 [L1 band]).

In plasmid pDB3, there is a distance of about 800 bases between the p_L promoter and the start of the *trkA* gene. Moreover, at least one *tem* promoter and the presumed *trkA* promoter are also located between these two sites and might enhance transcription (Fig. 4). However, at 28°C, no synthesis of the TrkA protein could be detected (Fig. 5, lane 1; Fig. 7, lane 1), indicating that the overproduction of the protein observed at 42°C (Fig. 5, 7, and 8) was primarily the result of enhanced transcription initiated by the λ p_L promotor. Despite the remoteness of this promoter, the TrkA protein was overproduced to such an extent that it became the major cellular protein (Fig. 5). Our present investigations involve the removal of increasingly larger DNA fragments between the p_L promoter and the *trkA* gene to find whether the large overproduction of TrkA protein occurs because the p_L promoter is at some distance from the *trkA* gene.

The enormous overproduction of the TrkA protein directed by pDB3 had severe effects on the physiology of the cells. The optical density of the suspension became constant before the cells had produced maximal amounts of the protein (Fig. 5). Under these conditions, the cells became filamentous and formed contrast-poor intrusions, similar to those in cells that overproduce the F_1F_0 ATP-synthase complex (38). Our result that the TrkA protein cosedimented only in small amounts with the cytoplasmic membrane (Fig. 7 and 8) indicates that upon overproduction, this protein did not distribute randomly in the cytoplasmic membrane but instead formed aggregates, and it seems likely that these aggregates have structures similar to those formed by the ATP synthase (38), fumarate reductase (10, 39), or glycerol-3-phosphate acyltransferase (40) when overproduced.

The gene encoding the MW 17,000 protein is located between the *trkA* and *rplQ* genes (Fig. 1 and 2). This gene is not *tolM*, since our plasmids did not complement TolM activity (K. Hantke, unpublished observations). In fact, comparison of cotransduction frequencies of genes located in this area from references 6 and 35 indicates that *tolM* is located much closer to *rpsL* than is shown on the *E. coli* genetic map (1), placing *tolM* either among or beyond the cluster of ribosomal genes located at the 72.4-min area of the map. The MW 17,000 protein might be the RimE protein (Fig. 1A).

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