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Escherichia coli Produces a Cytoplasmic α-Amylase, AmyA

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In the gap between two closely linked flagellar gene clusters on the *Escherichia coli* and *Salmonella typhimurium* chromosomes (at about 42 to 43 min on the *E. coli* map), we found an open reading frame whose sequence suggested that it encoded an α-amylase; the deduced amino acid sequences in the two species were 87% identical. The strongest similarities to other α-amylases were to the excreted liquefying α-amylases of bacilli, with >40% amino acid identity; the N-terminal sequence of the mature bacillar protein (after signal peptide cleavage) aligned with the N-terminal sequence of the *E. coli* or *S. typhimurium* protein (without assuming signal peptide cleavage). Minicell experiments identified the product of the *E. coli* gene as a 56-kDa protein, in agreement with the size predicted from the sequence. The protein was retained by spheroplasts rather than being released with the periplasmic fraction; cells transformed with plasmids containing the gene did not digest extracellular starch unless they were lysed; and the protein, when overproduced, was found in the soluble fraction. We conclude that the protein is cytoplasmic, as predicted by its sequence. The purified protein rapidly digested amylose, starch, amylopectin, and maltodextrins of size G_6 or larger; it also digested glycogen, but much more slowly. It was specific for the α-anomeric linkage, being unable to digest cellulose. The principal products of starch digestion included maltotriose and maltotetraose as well as maltose, verifying that the protein was an α-amylase rather than a β-amylase. The newly discovered gene has been named *amyA*. The natural physiological role of the AmyA protein is not yet evident.

During an investigation of the flagellar genes of *Escherichia coli* and *Salmonella typhimurium*, we encountered a nearby open reading frame whose deduced product sequence suggested that it was that of a cytoplasmic α-amylase.

The only major cytoplasmic polysaccharide in these enteric bacteria is glycogen, which is laid down as an energy and carbon reserve, especially under conditions in which carbon is abundant but another major essential element, such as nitrogen, is limiting (27). Under normal growth conditions, glycogen represents only about 2.5% of the dry weight of the cell (26), but it can reach as high as 30% under conditions of carbon abundance and deprivation of another essential element, such as nitrogen (33). A possible role for a cytoplasmic α-amylase might therefore be in glycogen metabolism.

The enzymes responsible for glycogen synthesis in *E. coli* have been studied extensively; they are glucose-1-phosphate adenyltransferase, glycogen synthase, and 1,4-α-glucan branching enzyme. The corresponding structural genes (*glgC*, *glgA*, and *glgB*) are clustered at 76 min on the map (1, 2, 18).

Less is known about the genetics and enzymology of glycogen breakdown in *E. coli*. By analogy with mammalian systems, one might expect at least an α-glucan phosphorylase and a debranching enzyme. An *E. coli* protein with α-glucan phosphorylase activity was reported by Chen and Segel (5). Subsequently, two groups reported a gene, *glgP* (mapping adjacent to *glgA* in the *glg* cluster), that encodes an α-glucan phosphorylase (6, 39); its activity at high glucan concentrations was comparable to those of other well-studied phosphorylases. A debranching enzyme that was believed to be cytoplasmic was reported for *E. coli* (15), but its activity on glycogen as a substrate was low, and the gene encoding the enzyme was not identified. Maltodextrins are generated under conditions in which glycogen synthesis occurs, but the enzyme responsible for this activity has not been identified (8).

Many bacterial species (notably bacilli) excrete α-amylases into their environment to digest exogenous amylopectin and amylose; these have been studied extensively and are of commercial importance (37). The enteric bacteria, however, do not appear to excrete α-amylases (or enzymes in general).

There is an early report by Chambost and coworkers of the partial purification and characterization of a cytoplasmic α-amylase from *E. coli* (4); the enzyme was found to be much more active on starch as a substrate than on glycogen. *E. coli* also possesses a periplasmic α-amylase, encoded by the *malS* gene (11, 30). Its location and substrate specificity argue that it functions to break down maltodextrins that have entered the periplasm via the outer membrane maltoporin LamB but that are at or above the size limit (ca. G_6) of the MalK maltose transport system. *E. coli* also has a maltodextrin glucosidase, MalZ, that further breaks down maltodextrins in the cytoplasm (35).

We have identified a region of the chromosomes of *E. coli* and *S. typhimurium* that may be relevant to these issues, since it contains a gene encoding a cytoplasmic α-amylase. We report here the sequence of this gene, *amyA*, together with a characterization of the enzyme.

MATERIALS AND METHODS

Bacterial strains. *E. coli* K-12 strain DH5α was used for routine transformations. MGT7 (20) was used for transformation with derivatives of T7 polymerase vector pET11a. UH869 (13) was used as a minicell-producing strain.

Plasmids. Plasmid pIBI25 was from International Biotech-

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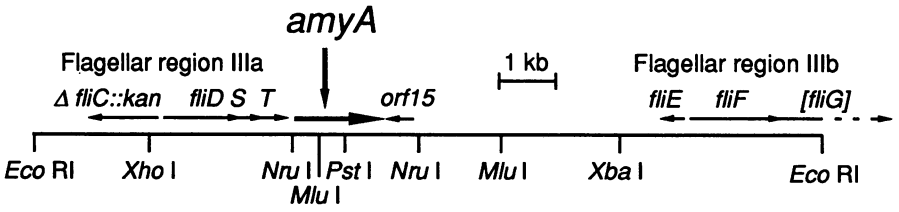

FIG. 1. Organization of the *E. coli* chromosome in the vicinity of the nonflagellar region between flagellar regions IIIa and IIIb (at 42 to 43 min), showing the location of the newly discovered α-amylase gene, *amyA*. The *Eco*RI fragment shown is the insert of plasmid pIK1001 (see the text); it extends from before flagellar gene *fliC* (which in this construction contains a partial deletion and has been disrupted by a *kan* gene cassette) to beyond *fliF*. *amyA* is preceded by the *fliDST* operon, the last in flagellar region IIIa, and is followed (in the opposite orientation) by an open reading frame, *orf15*, that encodes a 15-kDa protein of unknown function. Restriction sites referred to in the text are shown. *S. typhimurium* has a similar arrangement of flagellar region IIIa, *amyA*, *orf15*, and flagellar region IIIb.

nologies Inc. (New Haven, Conn.), Bluescript KS was from Stratagene (La Jolla, Calif.), and pHSG399 was from Takara Shuzo (Kyoto, Japan). T7 expression vector pET11a has been described (7). pIK1001 is a pBR322-based plasmid with an *E. coli* chromosomal fragment containing the *fliCDST* genes of flagellar region IIIa, all of the nonflagellar region between flagellar regions IIIa and IIIb, and the *fliEF* genes of flagellar region IIIb; the *fliC* gene contains a partial deletion and has been further disrupted by insertion of the *kan* gene (17) (Fig. 1). pIK1101 is a derivative containing the *Xho*I-*Xba*I portion of pIK1001 ligated into *Sal*I-*Xba*I-digested pHSG399. pIK1201 contains the *Nru*I-*Nru*I portion of pIK1001 inserted into pUC19. pOYA1 has an insert that contains all of the nonflagellar DNA between flagellar regions IIIa and IIIb of *S. typhimurium* (24). Other plasmids are described in Results.

Chemicals. Maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose, amylose, amylopectin, soluble starch, glycogen, cellulose, commercial α-amylase from *Aspergillus oryzae*, commercial β-amylase from malt, Na-K tartrate, and 3,5-dinitrosalicylic acid were all purchased from Sigma (St. Louis, Mo.).

Recombinant DNA techniques and sequencing. *Exo*III deletions were carried out with the Erase-a-Base system (Promega, Madison, Wis.). DNA fragments were purified with GeneClean II (Bio 101, La Jolla, Calif.). DNA sequencing was carried out as described previously (16) with the dideoxy chain termination method (29) and a sequencing kit from United States Biochemicals (Cleveland, Ohio). AmpliTaq DNA polymerase was from Perkin-Elmer-Cetus (Norwalk, Conn.). [^{35}S]dATP and [^{35}S]methionine were from Amersham (Arlington Heights, Ill.). Recombinant DNA techniques were done in accordance with standard protocols (28).

Expression of proteins in minicells. Minicell-producing strain UH869 was transformed with various plasmids, and minicells were prepared by the procedures of Bartlett and Matsumura (3) and Matsumura et al. (23). Proteins were labeled with [^{35}S]methionine, and the labeled proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was soaked in the fluor Na salicylate, dried, and autoradiographed with X-Omat film (Eastman Kodak, Rochester, N.Y.). Fractionation of cells into spheroplasts and periplasmic fluid was carried out as described previously (12).

Protein assay. Protein was estimated by use of a microassay kit from Bio-Rad (Richmond, Calif.).

Enzymatic assays. (i) Colony lysis assay. The colony lysis assay was performed as described previously (36). In brief, colonies were grown overnight, replicated on a nitrocellulose filter, and lysed by placement of the nitrocellulose filter on blotting paper saturated with lysis buffer (50 mM Tris-HCl [pH 7.5], 0.1% Triton X-100, 2 mg of lysozyme per ml) for 60 min at room temperature. The filter was placed on a starch-containing agar plate and incubated overnight at 37°C. The plate was then stained with iodine-iodide solution (0.1% I_2–0.4% KI).

(ii) Colorimetric assay for starch. Starch was assayed colorimetrically with iodine-iodide as described previously (32).

(iii) Colorimetric assay for reducing sugars. Reducing sugars generated from the digestion of various substrates were monitored in a spectral assay with 3,5-dinitrosalicylic acid as described previously (9). The amount of reducing sugar (maltose equivalents) liberated was estimated from a standard curve.

(iv) Thin-layer chromatography assay. The products of the reaction after incubation of the enzyme with different substrates at 5 mM concentrations were separated by thin-layer chromatography with Kiselgel 60 F254 plates (5 by 10 cm; Merck, Gibbstown, N.J.). A 2.5-μl sample was applied for each spot. The solvent used was butanol-ethanol-water (3:5:2 [vol/vol/vol]). The products of hydrolysis were visualized by spraying the plates with 20% H_2SO_4 and charring them for about 10 min at 100°C.

Purification of α-amylase. MGT7 cells (250 ml) transformed with an overexpression plasmid (see Results) were grown to an optical density at 600 nm of 0.6 to 0.8, and 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added. After 4 to 5 h of growth, the cells were centrifuged and washed once with 50 mM Tris-HCl buffer (pH 7.0). The cells were then suspended in 1 ml of the same buffer and sonicated (Branson model W140D Sonifier) for a total of ca. 3.5 min with seven 30-s bursts and cooling to 0°C between bursts. The sonicate was centrifuged at 10,000 × *g* for 60 min, and the supernatant was further centrifuged at 140,000 × *g* for 60 min. To the supernatant was added 0.25 vol of streptomycin sulfate (5% solution in 20 mM Tris-HCl [pH 8.0]), and the sample was kept in the cold for 2 h. It was then centrifuged for 10 min at 0°C and 10,000 × *g* to remove precipitated DNA, and the supernatant was stored at 0°C overnight. On the next day, the sample was again centrifuged, the supernatant was dialyzed against 50 mM Tris (pH 7.0), and the proteins were precipitated with ammonium sulfate in three steps, 0 to 30%, 30 to 60%, and 60 to 80%. The proteins from these steps were checked for α-amylase activity, 80% of which was present in the 30 to 60% fraction.

The pellet from this fraction was dissolved in 1.5 ml of 50 mM Tris-HCl buffer (pH 7.0)–0.2 M NaCl and loaded directly on a Sephadex G-150 column (2.5 by 50 cm; Pharma-

cia LKB, Piscataway, N.J.) equilibrated with the same buffer. The proteins were eluted with the same buffer and collected in 4-ml fractions. The starch-hydrolyzing activity of each fraction was measured, and active fractions were pooled together and loaded on a Fractogel EMD DEAE-650(S) high-pressure liquid chromatography column (Merck).

The proteins were eluted from this column with 5 mM Na phosphate buffer (pH 7.2) and an NaCl gradient of 0.25 to 0.8 M. The active α-amylase eluted at about 0.7 M NaCl. Excess salt was dialyzed away, and the protein was concentrated (Centriprep concentrator; Amicon, Danvers, Mass.) and finally stored in a 50% glycerol solution at −20°C.

Nucleotide sequence accession numbers. The sequences of the *E. coli* and *S. typhimurium amyA* genes have been deposited in GenBank under accession numbers L01642 and L01643, respectively.

RESULTS

Information derived from previous studies of the *fliDST* flagellar operons of *E. coli* and *S. typhimurium*. A recent analysis of flagellar region III of *E. coli* (located between 42 and 43 min on the chromosome) had established that it was really two regions, IIIa and IIIb, with an intervening region of at least 5.4 kb (between the first *Mlu*I site and the *Xba*I site shown in Fig. 1) that was unrelated to flagellation, since it could be deleted and the cells would still be fully motile (17). Strains with a deletion of the intervening region were also capable of normal growth on minimal media, indicating that this region did not contain any genes necessary for viability under these conditions (17). Between the 3′ end of the last known flagellar gene in region IIIa (the *fliT* gene, which ends the *fliDST* operon) and the first *Mlu*I site, there was a noncoding region of 77 bp and then a partial open reading frame that extended for 420 bp to that *Mlu*I site, the limit of the sequence available at that time (17). The *fliDST* operon in *S. typhimurium* had also been characterized (17) and was similar to that in *E. coli*: a noncoding region of 71 bp following *fliT* and a partial open reading frame that extended 846 bp to the limit of the then-available sequence (17). The deduced amino acid sequences of the N-terminal 140-residue fragments in the two species were 88% identical, arguing strongly that the partial open reading frames corresponded to real genes. When compared with the National Biomedical Research Foundation and SwissProt protein sequence data bases, the longer of the two deduced partial sequences (the 282-residue sequence of *S. typhimurium*) had highly significant optimized similarity scores against several bacillar α-amylases and weaker but still significant scores against barley α-amylases. We shall refer to this open reading frame in both species as *amyA* (amylase-alpha); the experimental justification for concluding that it is a gene encoding an α-amylase is described below. Optimal alignment of the *S. typhimurium* N-terminal AmyA sequence and the sequence of the precursor form of the excreted α-amylase of *Bacillus stearothermophilus* (14) revealed a high degree of identity (44%) and a displacement of the AmyA sequence that placed its N terminus in alignment with the N terminus of the mature form of the bacillar enzyme (see below). These results indicated that the gene encoded a cytoplasmic rather than a periplasmic or excreted enzyme.

Cloning and sequencing of *E. coli amyA*. The sequence of *amyA* through the first *Mlu*I site in Fig. 1 (a distance of 0.4 kb) was known. Anticipating (from the sizes of known α-amylase genes) that the complete gene would be about 1.5 kb long, we cloned the 3.3-kb *Mlu*I-*Mlu*I fragment from pIK1101 (see Materials and Methods) into pIBI25; the resulting plasmid, pMR1, had the insert in an orientation opposite from that shown in Fig. 1. Using vector restriction sites, we excised the *Bam*HI-*Hin*dIII fragment from pMR1 and cloned it into the corresponding sites in plasmid Bluescript KS to yield plasmid pMR2. These two plasmids were then used to generate *Exo*III deletions from both ends of the insert by use of the *Xho*I and *Kpn*I sites in the vector sequence of pMR1 and the *Hin*dIII and *Kpn*I sites in the vector sequence of pMR2. The sequence of the entire *Mlu*I-*Mlu*I fragment was obtained in both directions by use of the full-size fragment and *Exo*III deletions thereof.

E. coli amyA was found to be 1,485 bp long (Fig. 2) and was preceded by a strong, well-placed consensus ribosome binding sequence (GGAG, separated from the start codon by 7 bp). In the noncoding region between the end of *fliT* and the start of *amyA*, there was a stem-loop structure that might function as a transcription terminator of the *fliDST* operon (17). Possible candidates for the *amyA* promoter could be seen in both the *E. coli* sequence (Fig. 2) and the *S. typhimurium* sequence (data not shown). There was no sequence corresponding to the so-called MalT box (GGA[T/G]GA) that is found in the promoter region of operons that are part of the maltose regulon.

Following *amyA*, there was a stem-loop structure that could be a transcription termination sequence. This structure was followed by an open reading frame in an orientation opposite from that of *amyA*, with its 5′ end just before the second *Nru*I site shown in Fig. 1; this open reading frame was of sufficient length to encode a protein of about 15 kDa (data not shown) and will be referred to as *orf15*. The genomic organization of *E. coli amyA* and its surrounds is shown in Fig. 1.

We have also sequenced the corresponding region in *S. typhimurium*. The DNA sequence (not shown, but deposited in the GenBank data base) indicates that the partial open reading frame that was originally observed is indeed the 5′ end of a complete *amyA* gene.

Deduced sequence of the *E. coli* AmyA protein. The deduced sequence of *E. coli* AmyA corresponded to a protein of 495 residues and a molecular mass of 56 kDa; the *S. typhimurium* sequence was very similar (87% identity; Fig. 3). The similarity observed originally between the N-terminal region of AmyA and those of known α-amylases continued throughout its length. The highest similarity was seen to liquefying bacillar α-amylases. For example, AmyA and the liquefying α-amylase of *B. stearothermophilus* (14) were 42% identical throughout the entire length of AmyA (Fig. 3). The bacillar enzyme has an additional 36 residues at its N terminus in the deduced sequence and is cleaved after residue 34 during the excretion process (14). A comparison of AmyA and the liquefying α-amylase of *Bacillus amyloliquefaciens* (34) produced a similar result (41% identity and alignment with the mature rather than the precursor form; data not shown); these two liquefying α-amylases are themselves about 60% identical (25). Among amylases, even ones that are only distantly related, there are several highly conserved regions (25); these were well conserved in the AmyA sequence (Fig. 3).

The similarity of AmyA to other types of α-amylases was much lower; for example, except for the conserved amylase regions, it showed essentially no similarity to the saccharifying α-amylase of *Bacillus subtilis* (38) and little similarity to the periplasmic α-amylase, MalS, described by Schneider and coworkers (30).

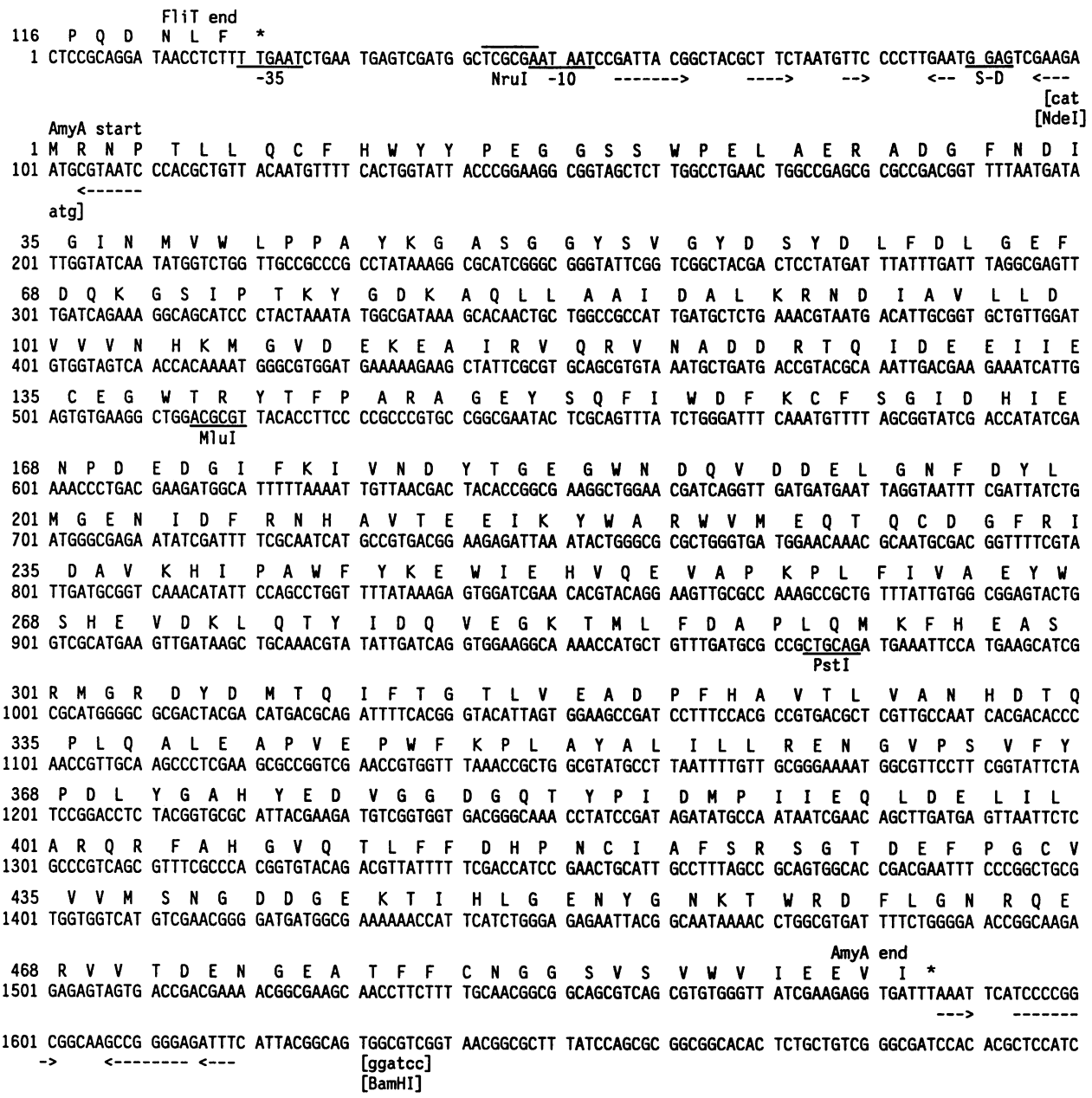

FIG. 2. Sequence of the *E. coli amyA* gene, along with the deduced amino acid sequence of its product. The sequence shown includes (i) the 3′ end of flagellar gene *fliT*, (ii) the noncoding region that contains the putative transcription termination sequence of the *fliDST* operon (arrows) and the putative promoter (−35 and −10, underlined) and ribosome binding site (S-D, underlined) of *amyA*, (iii) the *amyA* coding sequence, and (iv) the noncoding region beyond *amyA*, which includes the putative transcription termination sequence of *amyA* (arrows). Restriction sites mentioned in the text are shown; the bases shown in lowercase type below the wild-type sequence are mutations introduced to generate *Nde*I and *Bam*HI sites for cloning *amyA* into overexpression vector pET11a (see the text).

Identification of the *E. coli amyA* gene product. In a variety of plasmid constructions, the presence of the DNA corresponding to *E. coli amyA* correlated with the appearance of a protein with an apparent molecular mass of 56 kDa in radiolabeled minicell preparations. One such plasmid was pIK1201, which contained a 2.1-kb *Nru*I-*Nru*I fragment that extended from the noncoding region between *fliT* and *amyA* to about 0.5 kb beyond the end of *amyA*. As can be seen in Fig. 4, lane 2, this plasmid encoded a 56-kDa protein that was not encoded by the vector alone, pUC19 (lane 1). Plasmid pIK1101 (lane 3) contained a larger insert that included the *fliDST* operon as well as the *amyA* region; a protein with a slightly lower apparent molecular mass of 52 kDa, which corresponds to that of FliD (17), was seen, as was the 56-kDa protein. With plasmid pIK1110 (lane 4), in which the *Nru*I-*Nru*I portion of pIK1101 was deleted, the FliD band remained, but the 56-kDa band was absent. Deletion of a fragment of pIK1201 that extended from a *Pst*I site within the vector upstream of *amyA* to a *Pst*I site within *amyA* (at bp 974 in Fig. 2) yielded plasmid pMR3 and the loss

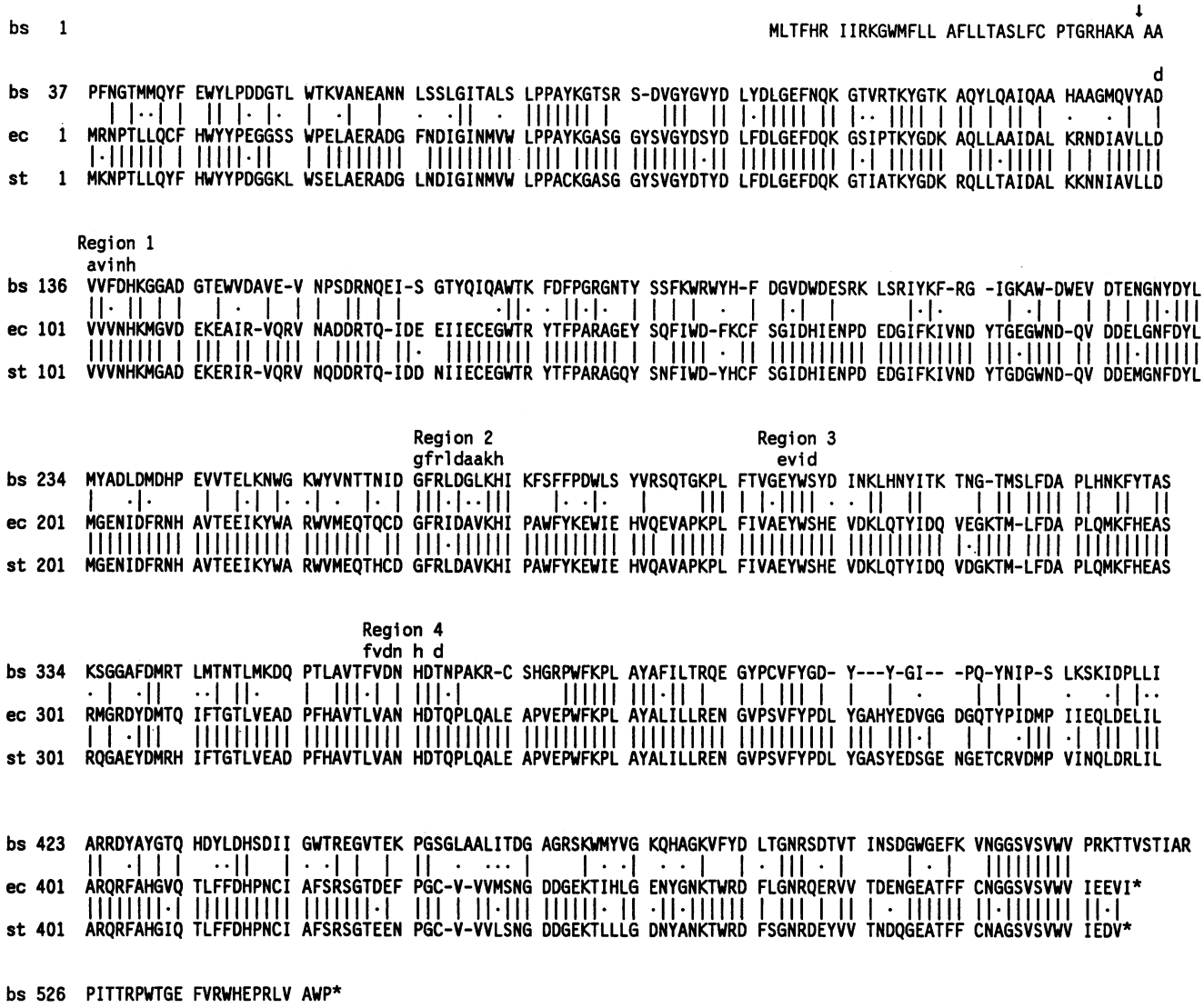

FIG. 3. Comparison of the amino acid sequences of the liquefying α-amylase of *B. stearothermophilus* (bs) (14), *E. coli* AmyA (ec), and *S. typhimurium* AmyA (st). The bacillar enzyme is excreted, with cleavage of the signal peptide at the site shown by the arrow. The alignment of the proteins by the Lipman-Pearson algorithm (22) places the N termini of the *E. coli* and *S. typhimurium* sequences and the bacillar mature sequence in close alignment. Identities are shown by vertical bars, and similarities (among I/L/V/M/F, F/Y, A/G, D/E, H/K/R, N/Q, or S/T) are shown by dots. Regions 1 to 4 (lowercase letters) are local consensus sequences that are highly conserved among α-amylases, even when the overall level of similarity is not high (25).

of the 56-kDa band (lane 6). We conclude from these results that the 56-kDa protein is the product of *amyA*.

Another protein, with an apparent molecular mass of 15 kDa, was encoded by pIK1201, pIK1101, and pMR3 but not pIK1110 (or any of the vectors alone) (Fig. 4), identifying it as the product of the open reading frame, *orf15*, adjacent to *amyA* (Fig. 1).

Starch-digesting activity of *E. coli* AmyA. DH5α cells transformed with plasmid pIK1201 (containing the *amyA* gene) were grown on Luria broth plates, the colonies were replicated on nitrocellulose filters, which were then laid on starch-containing agar plates, and iodine-iodide was added. Whereas in the case of bacilli that excrete α-amylases, this procedure results in clearing of the blue color of the complex in the vicinity of the colony, only traces of clearing were seen with DH5α(pIK1201). However, in a colony lysis assay, in which the cellular content of the colonies was released before the iodine-iodide was added, distinct clearing was observed with DH5α (pIK1201). Thus, the cellular content of DH5α(pIK1201) had significant amylolytic activity. Only a trace of clearing was observed with DH5α transformed with vector pUC19 alone, indicating that the chromosomal level of expression of *amyA* or other amylolytic genes was low.

Localization of *E. coli* AmyA in minicells. To confirm that AmyA was not being exported across the cell membrane, we prepared radiolabeled minicells and converted them into spheroplasts by using lysozyme-EDTA. AmyA was retained

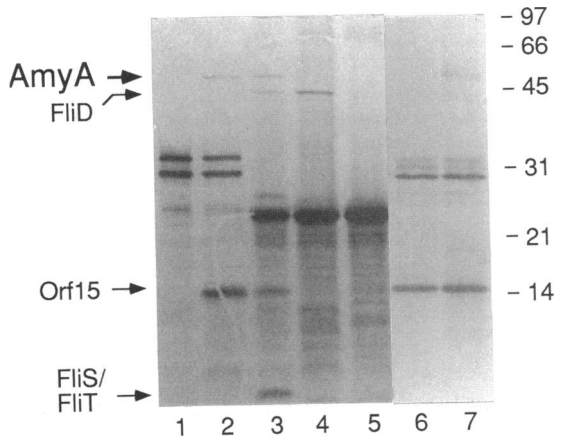

FIG. 4. Minicell analysis showing the products encoded by various plasmids. Lanes: 1, pUC19 vector; 2, pIK1201, a pUC19-based plasmid containing the *Nru*I-*Nru*I fragment shown in Fig. 1; 3, pIK1101, a pHSG399-based plasmid with an insert that includes the *fliDST* operon as well as the *amyA* region; 4, pIK1110, an *Nru*I deletion derivative of pIK1101; 5, pHSG399; 6, pMR3, a derivative of pIK1201 in which the 5′ two-thirds of the *amyA* sequence has been lost as a result of a *Pst*I deletion; 7, pIK1201. The assignments of bands to AmyA, FliD, FliS, FliT, and Orf15 are indicated. The positions of molecular mass markers (in kilodaltons) are shown at the right.

with the spheroplast fraction (Fig. 5, lane 2), whereas known periplasmic proteins, such as the mature form of the plasmid-encoded β-lactamase, were released by the treatment.

Overproduction of *E. coli* AmyA. In the minicell experiments described above, the AmyA band was fairly weak (much weaker than that of the *orf15* product [Orf15], for example), suggesting a low level of expression of the *amyA* gene under the control of its natural promoter. For further characterization of the protein, the gene was therefore transferred into an overexpression system. Using mutagenic primers and the polymerase chain reaction, we amplified a

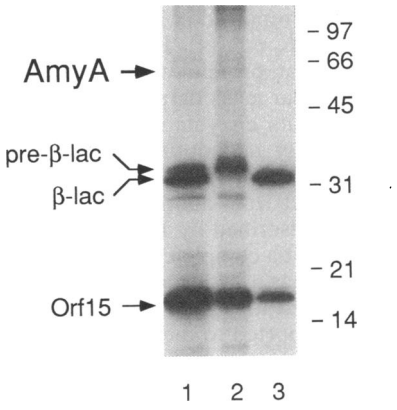

FIG. 5. Localization of AmyA in minicells. Minicells transformed with pIK1201 were radiolabeled and treated with lysozyme-EDTA to release the periplasmic content, and the spheroplasts were pelleted. Lanes: 1, whole minicells; 2, spheroplast fraction; 3, periplasmic fraction. AmyA was found almost exclusively in the spheroplast fraction. The precursor (pre-β-lac) and mature (β-lac) forms of the plasmid-encoded β-lactamase were found, as expected, in the spheroplast and periplasmic fractions, respectively. Molecular mass markers are as defined in the legend to Fig. 4.

TABLE 1. Purification of AmyA[a]

Sample or step of purification	Total activity[b]	Sp act[c]
Crude cell extract	200	25
Streptomycin sulfate supernatant	170	25
Ammonium sulfate (30 to 60%) fraction	120	30
Sephadex G-150 gel filtration	160	82
DEAE chromatography	50	120

[a] The reaction mixture contained 0.5% (wt/vol) amylose in 1 ml of 90 mM Na phosphate buffer (pH 7.2)–0.1 M NaCl–1 mM $CaCl_2$ at 45°C.
[b] Expressed as milligrams of maltose equivalents liberated $minute^{-1}$ $milliliter\ of\ extract^{-1}$.
[c] Expressed as milligrams of maltose equivalents liberated $minute^{-1}$ $milligram\ of\ protein^{-1}$.

1,561-bp fragment of DNA, introducing an *Nde*I site at the start codon of *amyA* and a *Bam*HI site shortly after its stop codon (Fig. 2). The *Nde*I-*Bam*HI subfragment was then inserted into the corresponding sites of the T7 expression vector, pET11a (7), such that *amyA* was now under the control of the T7 gene-10 promoter and ribosome binding site.

Transformed MGT7 cells were checked by the colony lysis assay, and several colonies were found to produce a large clearing zone. The plasmid from one such clone was isolated and named pMR6; its construction was verified by restriction enzyme analysis and by sequencing of the end points of the gene. When MGT7 cells transformed with pMR6 were grown in Luria broth to an optical density at 600 nm of 0.8 and then induced with 0.4 mM IPTG, a 56-kDa protein was evident within 1 h, whereas this band was completely absent even after 3 h in cells transformed with pET11a. In the colony lysis assay, the extent of clearing was considerably greater for MGT7 cells transformed with pMR6 than for DH5α cells transformed with plasmid pIK1201, in which *amyA* is under the control of its own promoter.

Overproducing cells were sonicated and fractionated by high-speed centrifugation. The supernatants from these and from control cells were then assayed by a colorimetric iodine-iodide assay. The results confirmed that the overproduction resulted in greatly increased amylolytic activity (by about a factor of 130; data not shown). They also confirmed that the activity was in the soluble fraction.

Purification of *E. coli* AmyA. The overproduced AmyA was purified as described in Materials and Methods, and the amylolytic activity at each stage was monitored with an assay that measures the release of reducing sugars (Table 1). SDS-PAGE of the protein at various stages of the purification process (Fig. 6) established that the protein was close to homogeneous, except for a minor band at a slightly lower apparent molecular mass, following the final stage of DEAE column fractionation.

Evidence that *E. coli* AmyA is an α-amylase. Starch was subjected to the action of various enzymes, and the products were analyzed by thin-layer chromatography. Commercial β-amylase (Fig. 7, lanes 4 and 5), as expected, digested starch by exoglycosidic cleavage of maltose units. AmyA (lanes 2 and 3) and commercial α-amylase (lane 6) cleaved randomly to produce a spectrum of products that included maltose, maltotriose, and maltotetraose. This result established that AmyA was indeed an α-amylase.

Activity of *E. coli* AmyA on various polysaccharides and oligosaccharides. Next we examined how effective AmyA was in digesting α-glucans with various degrees of branching, using the colorimetric assay for released reducing sug-

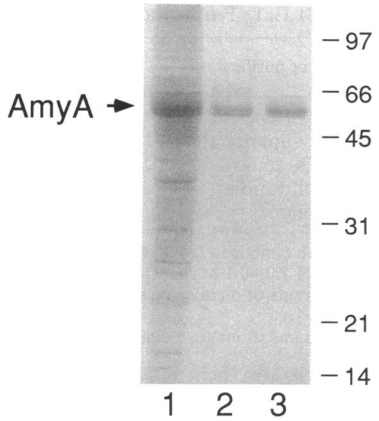

FIG. 6. Coomassie-stained SDS-polyacrylamide gels showing AmyA at various stages of the purification process. Lanes: 1, supernatant from streptomycin sulfate precipitation of the cytoplasmic fraction of sonicated DH5α(pMR6) cells; 2, material following ammonium sulfate fractionation and gel filtration; 3, material following DEAE chromatography. Molecular mass markers are as defined in the legend to Fig. 4.

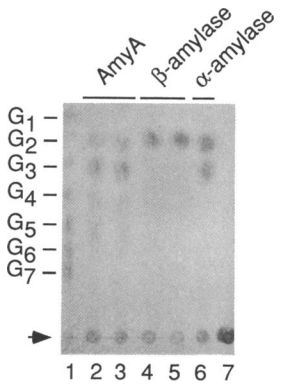

FIG. 7. Thin-layer chromatography showing the digestion of starch by AmyA and other enzymes. Lanes: 1, oligosaccharide standards (G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose; G6, maltohexaose; G7, maltoheptaose); 2 and 3, starch digested by AmyA for 2 and 4 h, respectively; 4 and 5, starch digested by commercial malt β-amylase for 2 and 4 h, respectively, with maltose as the sole product; 6, starch digested by commercial α-amylase from *A. oryzae* for 30 min; 7, undigested starch. The arrow indicates the position at which samples were loaded.

ars. The results established that the linear α-glucan amylose was the most effective substrate, with a catalytic constant (k_{cat}) of 690 s^{-1} (measured in terms of maltose equivalents liberated); this value is similar to the value of 660 s^{-1} obtained for the commercial α-amylase from *A. oryzae*. The lightly branched α-glucan amylopectin was somewhat less effective (k_{cat} = 330 s^{-1}), and starch, a mixture of amylose and amylopectin, was intermediate (k_{cat} = 530 s^{-1}). Glycogen, a highly branched α-glucan, was a poor substrate (k_{cat} = 1.6 s^{-1}) that required high enzyme concentrations and prolonged incubation before any digestion was detected. Cellulose, a β-glucan, was not digested at all.

The time course of appearance of products of the digestion of various oligosaccharide substrates was examined by thin-layer chromatography. Maltotetraose was not digested by AmyA (Fig. 8a), even after overnight incubation. Maltopentaose was a rather poor substrate, yielding maltotriose and maltose in appreciable amounts only after about 5 h (Fig. 8b). Maltohexaose yielded appreciable amounts of maltotetraose, maltotriose, and maltose at the earliest time point examined, 30 min (Fig. 8c); the amounts increased with time, digestion being essentially complete after 5 h. Maltoheptaose was an even better substrate, with digestion to maltopentaose and maltose being complete by 30 min (with traces of maltotetraose and maltotriose) and further digestion of the maltopentaose to maltotriose and maltose proceeding more slowly (Fig. 8d).

Temperature, pH, and salt characteristics of *E. coli* AmyA. The activity of AmyA was measured as a function of pH and temperature. At 45°C, the activity was maximal at about pH 7.2. At that pH, activity increased with temperature up to 45°C and declined steadily thereafter. Salt stimulated the enzyme; the activity was about twofold higher in the presence of 0.7 M NaCl than in its absence; and $CaCl_2$ at 1 mM also had a stimulatory effect, increasing the activity by about 30%.

DISCUSSION

***E. coli* produces a cytoplasmic α-amylase, AmyA.** We have found that *E. coli* synthesizes an enzyme whose activity defines it as an α-amylase. The name *amyA* has been given to the gene encoding this enzyme. A homologous gene was found to exist in *S. typhimurium* and, while we did not characterize the *S. typhimurium* enzyme biochemically, its high degree of amino acid identity to the *E. coli* enzyme indicates that it will have the same enzymatic activity.

The amino acid sequence of AmyA, when compared with those of α-amylases that are excreted by other bacterial species (and so have a cleaved leader peptide), led to the prediction that it would be a cytoplasmic enzyme. This prediction was validated by the absence of significant enzymatic activity in the vicinity of whole-cell colonies but the presence of such activity if the cells within the colonies were first lysed. It was further supported by minicell fractionation experiments and by the segregation of enzymatic activity to the supernatant fraction of sonicated cells.

Chambost et al. reported in 1967 the finding of a cytoplasmic α-amylase in *E. coli* (4). It is difficult to say retrospectively whether that protein was in fact AmyA, since no genetic or molecular mass data were described. It may well have been, since its enzymatic properties with respect to substrate specificity and optimum parameters for activity were similar to those of AmyA.

AmyA is distinct from another *E. coli* α-amylase, MalS, which has been described by Boos and colleagues (11, 30). The *malS* gene is at a completely different map location, and the MalS protein has a deduced amino acid sequence that is only weakly related to that of AmyA, has a cleaved signal peptide, and is exported to the periplasm. Whereas the role of MalS appears to be the digestion of periplasmic maltodextrins, the role of AmyA must be in the digestion of cytoplasmic polysaccharides or oligosaccharides. The α-amylase activity released by certain outer membrane-defective mutants of *E. coli* (32) may have been derived from MalS, since that study did not distinguish between the release of periplasmic and cytoplasmic contents, and there did not seem to be general loss of cytoplasmic material.

Physiological role of the enzyme. The present study confined itself to a characterization of the *amyA* gene and a biochemical characterization of its product. Extension of the

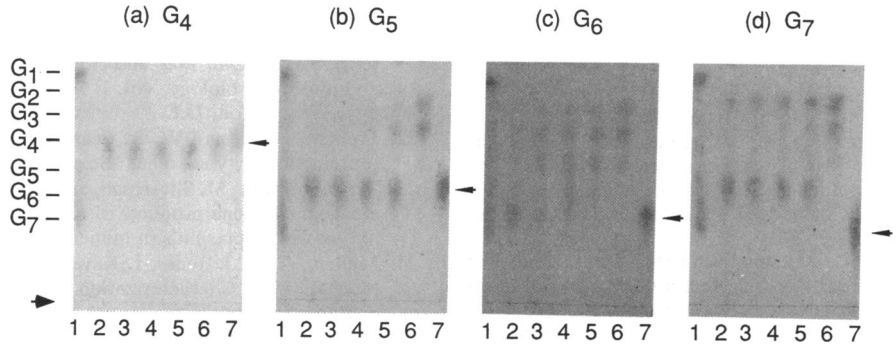

FIG. 8. Thin-layer chromatography of various maltodextrins digested by AmyA: a, maltotetraose (G_4); b, maltopentaose (G_5); c, maltohexaose (G_6); and d, maltoheptaose (G_7). In each panel, lane 1 contains oligosaccharide standards, as in Fig. 7, lane 7 contains the substrate without the addition of enzyme (small arrows), and lanes 2 to 6 contain the products after 0.5, 1, 2, 5, and 12 h of digestion with AmyA, respectively. The large arrow indicates the position at which samples were loaded.

analysis into the area of physiology is needed in the future, but it is worth considering what the available biochemical data suggest in terms of a physiological role for AmyA.

Among polymeric α-glucans, those that were either linear or lightly branched (amylose or amylopectin) were the most rapidly digested by AmyA; they are unlikely, however, to be the natural substrates for AmyA, since they are not known to exist in the cytoplasm of *E. coli*. AmyA was also effective in digesting linear oligomeric α-glucans (maltodextrins) of size G_6 or larger. The upper size limit of maltodextrins that can be transported into the cell by the MalK system is about G_6 or G_7 (21), so AmyA could participate in the digestion of these larger oligosaccharides. Arguing against this hypothesis are the facts that the *amyA* gene lies outside the known *mal* gene regions and that its upstream region lacks the MalT box characteristic of the genes of the *mal* regulon. Also, there already exist pathways for the catabolism of maltodextrins, via the phosphorolysis reaction mediated by maltodextrin phosphorylase, MalP, the disproportionation reaction mediated by amylomaltase, MalQ (31), and the release of glucose moieties from the reducing end of small maltodextrins by MalZ (35).

Glycogen proved to be a rather poor substrate for AmyA. Despite this result, we still consider it the most likely natural substrate, since it is the only polysaccharide present in appreciable amounts in the cytoplasm of *E. coli*. It might be that the optimum conditions for activity were not attained; for example, the enzyme might need to be located inside the glycogen particles to be effective, or it might require modification or the participation of a cofactor. Also, it might work more effectively in combination with α-glucan phosphorylase and a debranching enzyme than in isolation.

A hypothesis involving the generation of primers for glycogen synthesis was put forward by Chambost et al. (4) for the function of the cytoplasmic α-amylase of *E. coli* that they described (which, like AmyA, was a poor enzyme for digesting glycogen). During glycogen synthesis, chain elongation uses the enzyme glycogen synthase and the substrates ADP-glucose (*E. coli*) or UDP-glucose (mammals) plus α-glucan (possibly linked to some other molecular structure). The elongation reaction is written as G_n + ADP-glucose → G_{n+1} + ADP. The question then arises of how small G_n can be and still be an effective substrate for glycogen synthase. For the *E. coli* enzyme, glucose does not support the reaction even at 1.4 M, and maltose supports it at less than 10% of the rate achieved by oligosaccharides such as maltotetraose and larger oligosaccharides (10). The latter are quite good substrates, indicating that, at least in *E. coli*, pure oligosaccharides could act as primers for glycogen synthesis. Where would they be derived from? If exogenous oligosaccharides were available, they could be taken up into the periplasmic space via the maltoporin LamB and (shortened, if necessary, by MalS action) then transported to the cytoplasm via the MalK system. In the absence of an exogenous source, however, the cell would have to generate them. There is no known anabolic pathway for synthesizing oligosaccharides from glucose (the maltodextrin phosphorylase reaction of MalP, although reversible, cannot operate on molecules smaller than about G_4 or G_5). They might, however, arise from catabolism; existing cellular glycogen might act as a (poor) substrate for AmyA to yield oligosaccharides that could then act as the source of primers for the synthesis of many more molecules of glycogen.

Interestingly, Ehrmann and Boos (8) reported that *E. coli* generates maltodextrins in a manner that is dependent on glycogen synthesis. The enzyme responsible for this maltodextrin production has not been identified but could be AmyA.

Relationship between the *amyA* gene and its surrounds. Although *amyA* is immediately adjacent to a cluster of flagellar genes, this location does not seem to reflect any role of AmyA in flagellar assembly or function, since severe truncation of *amyA* still leaves cells fully flagellate, motile, and chemotactic (17). Also, the putative promoter of *amyA* does not resemble flagellum-specific promoters (19). As has been discussed elsewhere (17), an originally contiguous set of flagellar genes (those now in regions IIIa and IIIb) was probably disrupted by a chromosomal rearrangement that happened to juxtapose flagellar genes and a gene encoding an α-amylase. In *S. typhimurium*, flagellar region III is disrupted in the same way, with the intervening region commencing with a gene homologous to *E. coli amyA*. Thus, the presumed chromosomal rearrangement is a fairly old one, having occurred prior to the speciation of *E. coli* and *S. typhimurium*.

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