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BACTERIAL PERIPLASMIC PROTEINS AS EXTRACELLULAR SIGNALS FOR *ESCHERICHIA COLI*: GALACTOSE-BINDING PROTEIN STIMULATES PHOSPHATE INCORPORATION INTO NUCLEOTIDES*

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In multicellular organisms, hormones and other messenger molecules serve as mediators of cell-to-cell interactions which in turn play important roles in a variety of complex cellular processes such as differentiation, growth regulation, and specialized tissue functions. Accordingly, our present understanding of hormone-mediated mechanisms stems by and large from studies of higher organisms (1). However, there is well documented evidence that some unicellular eukaryotes and prokaryotes utilize hormone-like molecules as enviromental and intercellular signals (2, 5). For instance, in the yeast *Saccharomyces cerevisiae* small polypeptides (11, 13 amino acids) produced by cells of a given mating type induce specific changes among neighboring cells of the opposite type (4). In bacteria, specific polypeptides present in cultures of *Streptococcus faecalis* induce aggregation and increase the mating frequency of plasmid-carrying isogenic bacteria (5).

In this report we present evidence that cells of *Escherichia coli* and other related species react without measurable delay to the extracellular presence of periplasmic bacterial proteins by increasing up to 60-fold the

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was stopped with 0.09 ml of 7.8% trichloroacetic acid followed by 5 μl of 0.2 M KH_2PO_4 and 5 μl BSA 4 mg/ml. The precipitate formed was pelleted by a 2-min centrifugation (Fisher Micro-centrifuge model 235B), and a 0.1 ml sample from the supernatant was removed and mixed with 0.1 ml of a 5% water suspension of acid-washed activated charcoal (Norit-A) containing 0.05% sodium azide. The mixture was allowed to stand at room temperature for 5 min with occasional shaking, then filtered through a glass filter (Whatman GF/A). After four washes with 1 ml of 50 mM KH_2PO_4 each, the filter was dried for 30 min at 160°C, and its radioactivity was measured in a scintillation counter.

Nucleotide analysis. P^{32}-labeled nucleotides were analyzed by two-dimensional thin layer chromatography in poly(ethylene)imine cellulose (14, 15). To this end, samples taken at intervals during the assay were centrifuged, and the cells were extracted with 0.15 ml of 1 M formic acid at 0° for 30 min. After extraction, the cells were removed by centrifugation, and the supernatant was treated with sodium tungstate and procaine to eliminate inorganic phosphate (16). The resultant supernate was neutralized with triethanolamine (14), and spotted on TLC plates (Macherey-Nagel from Brinkmann Instruments). Purified ^{32}P-labeled nucleotide were identified by two-dimensional chromatography using radioactive samples mixed with authentic nucleotides, and by analyses after treatment with phosphodiesterase, alkaline phosphatase and sodium periodate (14). For quantitative measurements, the radioactive spots were located by autoradiography, cut off from the TLC plate, and their radioactivity was measured in a scintillation counter.

RESULTS

Effect of GBP on $^{32}P_i$ incorporation into nucleotides. Cells exposed to GBP for a short time period (5-20 min) incorporated 7 to 9-fold more radioactive inorganic phosphate into nucleotides than control cells without protein (Table 1). The rate of incorporation was proportional to bacteria concentration (in the range 2×10^6 - 2×10^7 per assay), and proceeded linearly without any noticeable lag for at least 20 min.

As shown in Table 2, the incorporation rate was proportional to GBP concentration, and it reached a maximal value in the presence of about 4×10^{-6} M GBP. Kinetic experiments in which the effect of varying phosphate concentration on the reaction rate was measured showed an apparent K_m of 8.3×10^{-6} M, and a maximal rate (apparent V_{max}) of 2020 nucleotides synthesized per cell per second (data not shown). The actual measured average rate at 4×10^{-6} M phosphate was 655 nucleotides per cell per second.

TABLE 3

EFFECT OF PROTEINS ON INCORPORATION OF $^{32}P_i$ INTO NUCLEOTIDES*

Addition	Radioactivity (cpm)	Ratio
None	59	1.0
Cytochrome C	29	0.5
Human serum albumin	49	0.8
Hemoglobulin	83	1.4
Alpha Chymotrypsinogen A	106	1.8
Gelatin	139	2.4
Bovine serum albumin	220	3.7
Egg albumin	339	5.7
oppA protein	660	11.2
GBP	1417	24.0

*Incorporation of radioactive phosphate into nucleotides was measured as indicated in Material and Methods. The assay mixtures contained 4×10^{-6} M of the indicated proteins. The data were corrected by 221 cpm, the average radioactivity of controls without bacteria. The ratio between values obtained with and without the respective protein is shown.

Specificity of the reaction. The effect of substituting unrelated proteins for GBP is shown in Table 3. With exception of the *oppA* protein, none of the proteins tested significantly stimulated $^{32}P_i$ incorporation. It should be noted that bovine serum albumin was ineffective even at concentrations 10-fold higher than that used for routine assays with GBP.

To test whether GBP stimulation was related to the galactose-binding properties of the protein, the effect of adding D-galactose to the reaction was examined. The results showed that galactose had no effect in the signaling reaction. In addition, a GBP genetically defective in its galactose binding ability was found to give a signal comparable to that of normal GBP (data not shown). Together, these results indicate that GBP is not recognized by the cells through its galactose-binding site.

Some cations appeared to have significant effect on the signaling mechanism as illustrated in Table 4. Calcium inhibited the reaction at 10^{-4} M, while sodium required a 100-fold higher concentration to achieve the same inhibition level. Potassium was only slightly inhibitory at 10 mM. Manganese and magnesium did not affect the reaction to a significant extent, however the latter was included in the routine assays because it specifically increased the GBP-mediated $^{32}P_i$ incorporation by about 15%.

TABLE 5

GBP-INDUCED SIGNALING DURING THE BACTERIA GROWTH CYCLE*
RADIOACTIVITY - CPM (%)

Growth phase	Exp. 1		Exp. 2	
	With GBP	No addition	With GBP	No addition
Exponential (E1)	199 (9)	114	307 (14)	99
Exponential (E2)	747 (32)	82	502 (22)	55
Early stat. (ES)	1001 (44)	190	1171 (52)	241
Stationary (S)	2299 (100)	358	2247 (100)	321

*Incorporation of radioactive phosphate into nucleotides was measured as indicated in Materials and Methods using cells harvested at the indicated stages of their growth. The stages are defined as follows: In Exp. 1, the culture grew exponentially for about 135 min; samples E1, E2, ES, and S were taken at 73, 133, 205, and 325 min, respectively. In Exp. 2, the culture grew exponentially for about 190 min and the samples were taken at 107, 179, 250 and 370 min, respectively. The final GBP concentration in the assays was 3.8×10^{-6} M. The data were corrected by 238 cpm, the average radioactivity of controls without bacteria. The values in parentheses are percentage of the maximal value obtained in each experiment.

TABLE 6

GBP-INDUCED SIGNALING AMONG DIFFERENT BACTERIA*
RADIOACTIVITY (CPM)

Bacteria	Marker	With GBP	No addition
S185-726	F^-	2356	187
W6	F^+	3796	449
W1895	Hfr	3128	442
S183-726/F′ MS1054	F′	991	93
E-15 (*phoA*8-*ompF*627)	Hfr	1581	124
E. coli ML30		2943	684
Shigella dysenteriae 499		2206	470
Bacillus subtilis 23		611	305
Bacillus subtilis 168		299	93
Bacillus megaterium		71	51

*Incorporation of radioactive phosphate into nucleotides was measured as indicated in Materials and Methods. The final GBP concentration in the assay was 3.8×10^{-6} M. The data were corrected by 227 cpm, the average radioactivity of controls without bacteria. The first five bacterial strains indicated are *E. coli* K12.

the stationary phase. In addition, as the culture ages there is a concomitant decline of the signaling system;

iii) at present, the signaling mechanism has been observed in enteric bacteria, but not in soil bacteria.

Altogether these observations suggest that the signaling system could have significance when bacterial growth is limited by nutritional factors to communicate that surrounding bacteria are starving.

Considering that molecular mechanisms for peptide hormone action are still not completely understood, the *E. coli* system could provide a useful model to study because of the powerful genetic and biochemical approaches available for bacterial research. Furthermore, it would be of interest to compare the prokaryotic and eukaryotic hormone-controlled systems from the evolutionary point of view. In this respect, it is noteworthy that certain peptides extracted from bacteria mimic vertebrate hormones when tested *in vitro* using mammalian systems (see (20) for a review).

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