with 0.5-msec square pulses at 50 Hz for 5 minutes. Portions were assayed at the end of each successive control or stimulus period.

11. High potassium saline was identical to that described above, except that KCl was elevated to 100 m*M* and NaCl lowered to 40 m*M*. High potassium cobalt saline contained 5 m*M* $CoCl_2$ replacing $CaCl_2$.
12. The mean value was determined from four such release experiments; the range was 0.5 to 1.6 percent. Mean PLB extracted from those muscles was equivalent to 30 fmole of proctolin per muscle. Amounts of PLB recovered from the Sep-Pak before and after HPLC purification did not differ significantly.
13. The third thoracic ganglion containing the Ds motoneuron and the coxal depressor muscle 177d were removed intact from the animal and perfused in vitro with physiological saline. Ds was stimulated either intracellularly with a microelectrode placed in the cell body or extracellularly with a suction electrode placed on the nerve root. Muscle membrane potentials were recorded intracellularly with flexibly mounted microelectrodes, and the contractile response was measured with a force transducer (RCA 5734).
14. A "catch" property of coxal depressor muscle 177d described by M. Chesler and C. R. Fourtner [*J. Neurobiol.* **12**, 391 (1981)] may be related to the sustained tension we describe in this report.
15. Supported by NIH grant NS-16298 and NSF grant BNS-8202515, by the University of Chicago, and by the Zoecon Corporation. We thank K. G. Pearson for the RCA transducer.
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Synthesis and Secretion of the Plasmid-Coded Heat-Labile Enterotoxin of *Escherichia coli* in *Vibrio cholerae*

Abstract. *Both cholera toxin and heat-labile enterotoxin were made and secreted into culture supernatants by* Vibrio cholerae *containing the enterotoxin plasmid pCG86. Several regulatory mutations in* V. cholerae *that increased or decreased the synthesis of cholera toxin did not affect production of heat-labile enterotoxin. In contrast, a mutation in* V. cholerae *that interfered with the secretion of cholera toxin also decreased the secretion of heat-labile enterotoxin, indicating that they are processed by a common secretory pathway.* Vibrio cholerae *should be useful as a model system for analyzing the secretion of true extracellular proteins by Gram-negative bacteria.*

Vibrio cholerae and enterotoxigenic strains of *Escherichia coli* can cause diarrhea by producing enterotoxins that stimulate secretion of fluid and electrolytes in the small bowel (*1*). Cholera toxin (CT) and *E. coli* heat-labile enterotoxin (LT) are closely related in mode of action, subunit structure, and immunochemistry (*2–4*); the structural genes for CT and LT show strong homology although they are not identical in sequence (*5*), and they are organized into similar operons (*6, 7*). Nevertheless, several differences in the genetic control of these heat-labile enterotoxins have been reported. Most of the CT produced by *V. cholerae* is secreted (*8*), but most of the LT synthesized by *E. coli* remains cell-associated (*4, 9, 10*). The A and B subunits of LT are synthesized as separate polypeptides (*6, 11*), but one preliminary study suggests that the subunits of CT might be derived from processing of a larger precursor polypeptide (*12*). The structural genes for CT appear to be chromosomal in *V. cholerae* (*7*), but the genes for LT are encoded on plasmids in *E. coli* (*5, 13*).

We constructed bacterial strains that produced both CT and LT by transferring the enterotoxin plasmid pCG86 (*14*) from *E. coli* into *V. cholerae*. By comparing the synthesis and secretion of CT and LT in isogenic strains of *V. cholerae* with wild-type or mutant alleles at loci that regulate toxinogenesis, we evaluated the roles of specific host functions on the expression of the homologous structural genes of CT and LT. We used specific competitive-binding radioimmunoassays to measure each enterotoxin antigen in the presence of the other toxin (Fig. 1).

Our analyses of the synthesis and secretion of CT and LT in wild-type and mutant strains of *V. cholerae* containing plasmid pCG86 are summarized in Table 1. All strains of *V. cholerae* containing plasmid pCG86 produced detectable amounts of LT antigen, whereas none of the control strains lacking pCG86 produced the antigen. Therefore, the structural genes for LT can be expressed in *V. cholerae*. In cultures of wild-type *V. cholerae* 569B (*8*) and of 569B containing plasmid pCG86 [569B(pCG86)], almost all of the CT was in the culture supernatant. Approximately 95 percent of the LT produced by 569B(pCG86) was also in the culture supernatant. Extracellular protein presented less than 4 percent of the total protein in these cultures. Therefore, both CT and LT are secreted by *V. cholerae*. In contrast, studies of the expression of cloned CT structural genes in *E. coli* showed that CT (*7*), like LT (*4, 9, 10*), remained cell-associated in *E. coli*.

Synthesis of CT is controlled by regulatory genes in at least two different loci on the genetic map of *V. cholerae*, and different alleles at these loci are associated with hypertoxinogenic or severely hypotoxinogenic phenotypes (*15, 16*). When plasmid pCG86 was present in strain M13, a severely hypotoxinogenic mutant of 569B (*8*), LT antigen was produced and secreted in amounts comparable to those observed with 569B(pCG86). Strain CA401 is a wild-type, virulent strain of *V. cholerae* that produces much less CT than 569B does (*17*). HV131 is a highly toxinogenic mutant of CA401 that produces CT in yields comparable to those produced by 569B; HV133 is a hypotoxinogenic mutant of CA401. Both HV131 and HV133 were isolated from survivors of nitrosoguanidine mutagenesis (*18*) of CA401 that exhibited altered toxin production in a toxin-dependent plate hemolysis assay (*19*). Although the total amount of CT produced by HV131 was approximately 100 times that produced by CA401 or

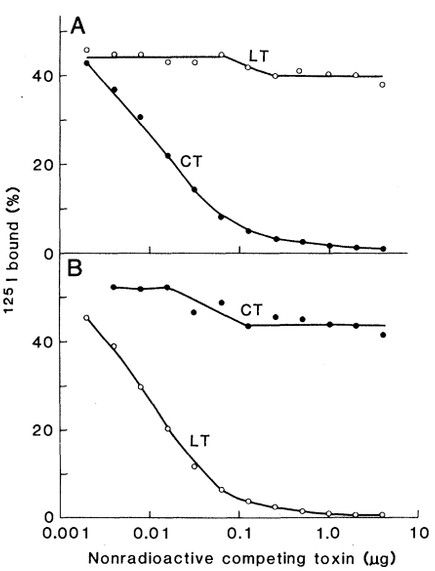

Fig. 1. Specific radioimmunoassays for cholera enterotoxin (A) and *E. coli* heat-labile enterotoxin (B). Purified cholera enterotoxin (*CT*) from *V. cholerae* 569B (*22*) and purified heat-labile enterotoxin (*LT*) from *E. coli* strain HE12 (*4, 10*) were labeled with $Na^{125}I$ (*23*) and used in competitive-binding radioimmunoassays (*24*). Each reaction mixture contained ^{125}I-labeled toxin, rabbit antiserum to the homologous purified toxin, and unlabeled CT (●) or LT (○) as the competing antigen; immune complexes were absorbed on protein A–bearing *Staphylococcus aureus*, and the amount of ^{125}I bound was determined. Most of the antibodies in each immune serum were directed against the unique determinants of the homologous toxin antigen. In the presence of a large excess of heterologous toxin antigen, there was only a slight decrease in the amount of ^{125}I bound in the assay for CT (A) or for LT (B).

HV133, the amount of LT produced by HV131(pCG86) was very similar to that produced by CA401(pCG86) or HV133(pCG86). Bioassays performed with Y1 adrenal cell cultures (*20*) confirmed that the biologic activity of the enterotoxins in the culture supernatants was comparable to that expected on the basis of the amount of the enterotoxin antigens. All of our observations on production of CT and LT by plasmid-bearing mutants of *V. cholerae* 569B or CA401 supported the hypothesis that the regulatory functions defined by the mutations in these strains were specific for the control of CT production and did not affect the synthesis of LT.

The presence of pCG86 had small but detectable effects on the production of CT by *V. cholerae*. The yields of CT from *V. cholerae* strains containing pCG86 were increased in comparison with the same strains lacking pCG86. The significance of these changes requires further investigation. However, pCG86 did not reverse the altered *tox* phenotypes of mutants M13 and HV131, indicating that the plasmid did not provide regulatory elements that could completely replace the functions of the defective elements in these strains of *V. cholerae*.

Strain M14 is a mutant of *V. cholerae* 569B that is defective in its ability to secrete CT; more than 90 percent of the CT produced by M14 remains cell-associated (*8*). There were no significant differences in the amounts of extracellular protein or cell-associated protein in cultures of 569B and M14. When the distribution of LT and CT between extracellular and cell-associated compartments was determined for strain M14(pCG86), we found that more than 60 percent of LT and CT remained cell-associated, in contrast to the results with wild-type 569B(pCG86). Therefore, a common pathway affected by the mutation in strain M14 is involved in secretion of CT and LT by *V. cholerae*.

In summary, CT and LT are both secreted into the culture supernatant by *V. cholerae*; both remain cell-associated in *E. coli*; and the secretion of both enterotoxins in *V. cholerae* is affected by the mutation in M14. These findings indicate that the pathways for processing and secretion of CT and LT in *V. cholerae* differ from those in *E. coli*; it is extremely unlikely that the primary amino acid sequences of these enterotoxins are solely responsible for the differences in their cellular localization in *V. cholerae* and *E. coli*.

The secretion of CT and LT by *V. cholerae* is one of a limited number of examples (*21*) of export of proteins outside the cell envelope by Gram-negative bacteria. Most genetic studies of protein export in *E. coli* and related enteric bacteria have focused on the pathway for transport of proteins from the cytoplasm to the periplasmic space or outer membrane. The export of enterotoxins by *V. cholerae* outside the cell envelope may involve mechanisms of secretion that are not present in *E. coli*, and the availability of appropriate mutants should make *V. cholerae* useful as a model to study mechanisms for secretion of true extracellular proteins by Gram-negative bacteria.

Table 1. Amounts of CT and LT produced by wild-type and mutant strains of *V. cholerae* with or without the enterotoxin plasmid pCG86. 569B is a highly toxinogenic, wild-type strain; M13 is a hypotoxinogenic mutant of 569B; and M14 is a mutant of 569B that does not secrete CT (*8*). CA401 is a toxinogenic, wild-type strain of *V. cholerae* that produces a low yield of CT (*17*). HV131 is a highly toxinogenic mutant of CA401, and HV133 is a hypotoxinogenic mutant of CA401 (see text). Plasmid pCG86 was transferred by conjugation from *E. coli* KL320(pCG86) (*10*) to the *V. cholerae* strains listed. Equal volumes of overnight cultures of donor and recipient strains were inoculated onto meat extract agar plates (*25*) and incubated for 18 hours at 37°C. *V. cholerae* transconjugants were selected on minimal A agar plates (*26*) containing 0.25 percent glucose and 5 μg of tetracycline-HCl per milliliter. Assays for measurements of extracellular or cell-associated enterotoxin were performed with culture supernatants or with sonic extracts of bacteria, respectively. Cultures were grown overnight at 37°C in Syncase medium (*27*) with 0.25 percent glucose substituted for sucrose. Tetracycline-HCl (5 μg/ml) was added for strains containing pCG86. After centrifugation, culture supernatants were filtered through Millex filters (pore diameter, 0.45 μm; Millipore) and tested immediately for biologic activity. The remaining culture fluids were concentrated 15-fold with Minicon B-15 concentrators (Amicon) and stored at −20°C before being tested for extracellular enterotoxin antigens or protein. The bacteria were washed twice with buffer A (50 m*M* tris-HCl, *p*H 7.5, 1 m*M* EDTA, 3 m*M* NaN_3, and 200 m*M* NaCl), resuspended in one-tenth of the original culture volume in buffer A, and disrupted by sonication. Insoluble debris was removed by centrifugation at 27,000*g* for 30 minutes, and the bacterial extracts were sterilized by filtration through Millex filters and stored at −20°C before being tested for cell-associated enterotoxin antigens or protein. Enterotoxin antigens (CT and LT) were measured with competitive-binding radioimmunoassays (Fig. 1). Unknown samples were tested at several different doses, and concentrations of CT and LT were calculated by comparison with standard curves. Enterotoxin activity was measured with the Y1 adrenal cell assay (*20*) and was expressed as the equivalent concentration of purified CT or LT with comparable activity. Protein determinations were performed as described previously (*28*). All values are expressed as micrograms per milliliter of culture.

Bacterial strain	Protein (μg/ml)		Enterotoxin antigen (μg/ml)				Extracellular enterotoxin (μg/ml)
	Cell-associated	Extracellular	Cell-associated		Extracellular		
			CT	LT	CT	LT	
569B	590	13	*	*	0.75	*	2.5
569B(pCG86)	440	17	0.03	0.04	1.3	0.77	5.0
M13	610	8	*	*	*	*	0.005
M13(pCG86)	420	15	*	0.03	0.02	0.75	0.5
M14	570	10	0.39	*	0.03	*	0.01
M14(pCG86)	410	42	0.26	0.25	0.14	0.16	0.25
CA401	550	9	*	*	*	*	0.02
CA401(pCG86)	320	8	*	0.03	0.02	0.15	0.10
HV131	550	8	0.01	*	1.8	*	1.0
HV131(pCG86)	510	11	0.03	0.01	4.8	0.3	2.5
HV133	580	8	*	*	*	*	0.003
HV133(pCG86)	370	12	*	0.05	0.02	0.37	0.25

*Concentration was below the limit of sensitivity of the assay (approximately 5 ng per milliliter of culture in the radioimmunoassay; approximately 1 ng per milliliter of culture in the Y1 adrenal cell assay).

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A Cancer-Associated Lactate Dehydrogenase Is Expressed in Normal Retina

Abstract. *An unusual isozyme of lactate dehydrogenase, lactate dehydrogenase k, is found in high concentrations in cultured cells transformed by the Kirsten murine sarcoma virus and in many human cancer tissues. In experiments described here high levels of a lactate dehydrogenase k activity were detected in extracts of normal rodent retina. This activity had the same key properties as the human tumor isozyme, namely, a highly cathodic electrophoretic mobility and inhibition of enzymatic activity by oxygen and 5',5'-dipurinenucleoside tetraphosphates. Expression of this activity in the retina may be related to the high aerobic glycolysis characteristic of the retina, a metabolic feature shared with many tumors.*

Anderson and co-workers (*1, 2*) described an unusual lactate dehydrogenase (LDH), asp56/LDH_k (*3*), in cells transformed by the Kirsten sarcoma virus. This specific LDH activity is distinguished by (i) a highly cathodic electrophoretic mobility (*1, 2*); (ii) direct, reversible inhibition by physiological concentrations of oxygen (*1, 2*); (iii) direct, reversible inhibition by 5,5,-dipurinenucleoside tetraphosphates (*4*); and (iv) subunits of 56,000 daltons that are readily cleaved—without apparent loss of activity—into 35,000- and 22,000-dalton subunits (*4*). The enzyme can be induced in uninfected cultured fibroblasts by anaerobic shock (*4*) and is found in low but detectable levels in many normal tissues (*2, 4*). It is expressed at high levels in most human carcinomas and is found in serum of cancer patients (*4*).

The mammalian retina is essentially a portion of the brain specialized to transduce light into nerve impulses. The retina develops as an outgrowth of the brain walls. Early in development, before the prosencephalon is divided into telencephalon and diencephalon, the rudiments of the eye cup can be seen as a vesicle originating from the neural tube. Like other nervous tissues, the mature retina is in a nonproliferative state, that is, after differentiation is completed, no cell proliferation can be detected. The isolated retina, like many tumors, is characterized by a highly glycolytic metabolism under aerobic conditions (*5-7*). Photoreceptor cells are extraordinarily sensitive to high oxygen tension and glycolytic poisons such as iodoacetate (*6*). We report that an unusually high level of an asp56/LDH_k-like activity can be extracted from normal avian and mammalian retinas.

Retinas of mouse, rat, guinea pig, and chicken were dissected in toto and homogenized by Dounce homogenization in 0.01*M* tris buffer (*p*H 7.5) containing 0.001*M* dithiothreitol and 0.05 percent Triton X-100. Brain tissues were extracted in parallel as a control. The homogenates were centrifuged at 8000 rev/min for 45 minutes, and the supernatants were collected and assayed for asp56/LDH_k activity by using reverse-polarity electrophoresis on gels containing imidazole and borate (*1, 2*).

The amount of asp56/LDH_k-like activity extracted from rat and guinea pig retinas was far greater than the amount extracted from brain tissue from the same animals (Fig. 1A). With an integrating densitometer we determined that there was about 20 times more asp56/LDH_k-like activity in rat retina than in rat brain (200 versus 10 gel units per milligram). The concentration was approximately equal to the mean level in human carcinomas (*4*) and in fibroblasts transformed by Kirsten sarcoma virus (Fig. 1). The activity from rat retina migrated to a slightly different position than asp56/LDH_k activity from Kirsten sarcoma virus–transformed rat cells and asp56/LDH_k-like activity from guinea pig retina. In the retinas of both species at least two bands were observed that could represent either multiple isozymes of asp56/LDH_k or artifactual modifications. An unusually large amount of asp56/LDH_k-like activity was also extracted from mouse retina (Fig. 1B). Individual mouse, rat, and guinea pig retinal preparations were also assayed for

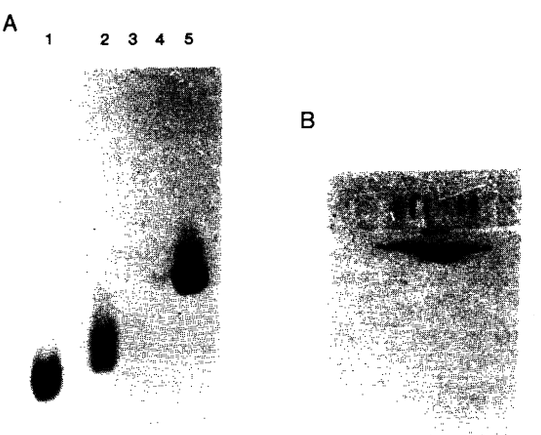

Fig. 1. Activity of asp56/LDH_k in normal rodents. (A) Lane 1, Kirsten sarcoma virus–transformed cells; lane 2, normal rat retina; lane 3, normal rat brain; lane 4, normal guinea pig brain; and lane 5, normal guinea pig retina. (B) Normal retinas pooled from C57BL/6J, BALB/c, and DBA mice. Equal amounts (30 μg) of total protein were added to each well. Gels stained in the absence of lactate showed no detectable activity.