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Transcription of the *Escherichia coli fliC* Gene Is Regulated by Metal Ions

ANGELINA GUZZO, CAROLINE DIORIO, AND MICHAEL S. DUBOW*

Department of Microbiology and Immunology, McGill University, 3775 University Street, Montreal, Quebec H3A 2B4, Canada

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***luxAB* gene fusions in the *Escherichia coli* genome were used to screen for clones displaying transcriptional changes in the presence of aluminum. One clone was found that contained a luciferase gene fusion in which transcription was increased in the presence of aluminum and which was subsequently shown to be induced by copper, iron, and nickel. Cloning of the metal-regulated gene, hybridization to the ordered phage λ bank of the *E. coli* chromosome, and sequencing of DNA adjacent to the *luxAB* fusion revealed that the insertion occurred within the *fliC* (*hag*) gene of *E. coli*. This gene encodes flagellin, the filament subunit of the bacterial motility organ, and is under the control of several regulatory cascades. These results suggest that environmental metals may play a role in the regulation of the motility potential of *E. coli* and that this bioluminescent gene fusion clone (or derivatives thereof) may be used to prepare a biosensor for the rapid detection of metal contamination in water samples.**

Toxic metals can have deleterious effects by blocking essential functional groups, displacing essential metal ions, or modifying the active conformation of biological molecules (5). Bacterial cells have evolved mechanisms to deal with elevated levels of these toxic environmental metals. The plasmid-borne heavy metal resistance operons found in many species of bacteria are a good example of this (14, 28).

Aluminum is a nonessential metal that is generally present in natural waters at concentrations ranging from approximately <0.03 to >0.67 μg/ml (3). Aluminum becomes more soluble with increasing acidity and can often be the most toxic element in acidic waters (6, 15). Several mechanisms have been postulated to explain aluminum toxicity (3, 27), including its potential to bind to DNA in *Rhizobium* spp. (15). Genetically programmed responses to elevated levels of aluminum in bacteria have not yet been defined. We chose to search for genes in the *Escherichia coli* chromosome that may play a role in cellular responses to elevated levels of aluminum in the environment. The rationale behind our approach was that genes (and their encoded products) that play a role in responding to elevated levels of aluminum could be induced (or repressed) at the level of transcription in the presence of the metal. To detect these transcriptional changes, a library of approximately 3,000 *E. coli* clones was created, each of which contained a random, single, chromosomally located insertion of a promoterless *luxAB* transcriptional reporter gene cloned within a truncated Tn*5* element (12). The gene product of the *Vibrio harveyi luxAB* genes, luciferase, is a mixed-function oxidase composed of α and β subunits that catalyzes oxidation of an aldehyde in a reaction that results in the emission of light (34). The amount of light produced by *E. coli* cells carrying the *luxAB* genes downstream from promoters is proportional to the amount of transcription of *luxAB* (8).

By monitoring changes in light emission of clones from the *E. coli luxAB* library in the absence and presence of aluminum, we identified a clone with a gene fusion in which transcription is induced in the presence of aluminum. We present evidence that transcription of this gene is also affected by other metals, and we identify this gene as *fliC* (formerly called *hag*), which encodes flagellin, the structural protein of the flagellar filament (21).

MATERIALS AND METHODS

Bacterial strains. *E. coli* DH1 [F⁻ *recA1 endA1 gyrA96 thi hsdR17* (r_k^-, m_k^+) *supE44 relA1*] (13) was used to prepare the library of *luxAB* transcription fusion clones (12). Strain NM522 [*supE thi* Δ(*lac-proAB*) Δ*hsd-5* (r_k^-, m_k^-) (F′ *proAB lacI*^{q}*ZΔM15*)] (11) was used for subcloning the chromosomal DNA from gene fusions originally present in strain DH1. Strain NM621 (*hsdR mcrA mcrB recD1009 supE44*) (32) was used for propagation of the λ phage *E. coli* chromosomal library prepared by Kohara et al. (17).

Media. Strains were routinely grown at 37°C in LB broth or on LB plates containing 1.5% agar (25) and supplemented with antibiotics when indicated. Tetracycline was used at final concentrations of 10 μg/ml in broth and 20 μg/ml in agar. Ampicillin was used at a final concentration of 40 μg/ml.

DNA manipulations. Plasmid DNA was purified by centrifugation to equilibrium in cesium chloride-ethidium bromide gradients (24). All restriction enzyme digestions and ligations were performed as previously described (30). Transformations were performed as described previously (23) and incubated at 37°C. Isolation of total cellular DNA was performed as previously described (12).

Construction of the *luxAB* gene fusion library. Construction of the *luxAB* gene fusion library has been described elsewhere (12). Briefly, the ColE1-based plasmid pRZ341-21 (16), containing a *tet* gene plus the *luxAB* genes within a left-end truncated Tn*5* element, was constructed and designated pFUSLUX. The plasmid pFUSLUX was introduced into an *E. coli* DH1 strain that contained an ampicillin-resistant plasmid, pTF421, which inhibits the replication of ColE1 plasmids via overproduction of RNA1 (10). Transformation on media containing tetracycline plus ampicillin selects for cells that contain the *luxAB* genes inserted into

* Corresponding author.

the chromosome at a single location. A library of approximately 3,000 such clones was created.

Construction of plasmid pAG5. Plasmid pAG5 was constructed by ligating 3.0 μg of *Bam*HI-cleaved total cellular DNA isolated from strain LF20111 to 1.0 μg of *Bam*HI-cleaved pUC119 (31). The ligation mixture was transformed into strain NM522 (11), and transformants were selected on tetracycline-containing LB plates (see Fig. 2).

Mapping with the Kohara library. The Kohara et al. (17) ordered λ library of the *E. coli* genome was propagated on *E. coli* NM621 and plated as described elsewhere (32). Transfer to nitrocellulose filters (Xymotech Biosystems Inc.; catalog no. 20570) was performed after the plaque lift procedure for DNA (24). The probe was prepared by isolating 1.0 μg of a 2.3-kb *Hin*dIII fragment from plasmid pAG5 and then labeled and hybridized by using the DIG DNA labeling and detection kit (Boehringer-Mannheim Canada Ltd.) according to the manufacturer's instructions.

Southern blotting and hybridization of probe. Ten micrograms of total cellular DNA was used per restriction endonuclease hydrolysis and then subjected to electrophoresis through a 0.75% agarose gel. Transfer of total cellular DNA to Hybond-N (Amersham Canada Ltd.) by the bidirectional method and hybridization were performed as described elsewhere (29). A sample (0.1 μg) of a 2.3-kb *Hin*dIII fragment was isolated from plasmid pAG5 and labeled by using the random-primed labeling method (9) with [α-^{32}P]dGTP (3,000 Ci/mmol; ICN Biomedicals Canada Ltd.).

DNA sequencing. Single-stranded phagemid DNA was prepared from plasmid pAG5 as described elsewhere (31). Dideoxy sequencing reactions with [α-^{35}S]dATP (500 Ci/mmol; Du Pont Canada Inc.) were performed with a Sequenase kit (U.S. Biochemical Corp.) according to the manufacturer's protocol. To determine the sequence at the junction between IS*50*R and the chromosomal DNA in pAG5, an oligonucleotide (5′AAGGTTCCGTTCAGGAC 3′) corresponding to bp 1497 to 1513 of IS*50*R (1) was synthesized (Sheldon Biotechnology Centre) and used as a primer. To determine the sequence at the *Bam*HI site in the chromosomal DNA, the *Bam*HI fragment of plasmid pAG5 (containing the *tet* gene, IS*50*R, and adjacent chromosomal DNA sequences) was cloned into the *Bam*HI site in the opposite orientation in the same vector (pUC119), and the −40 primer provided in the Sequenase kit was used.

Assay for luciferase-induced light emission. The light emission assay technique of Miyamoto et al. (26) was used. Briefly, 1-cm^2 patches of cells were grown overnight on an LB agar petri dish, placed upside down, and exposed to Kodak XAR-5 film at 23°C after the addition of 50 μl of decyl aldehyde (Aldrich Chemical Co., Inc.) to the cover of the petri dish. The X-ray films were developed after various times.

RESULTS

Identification of a gene whose transcription is increased in the presence of aluminum. Aluminum toxicity increases with decreasing pH due to its increased solubility at lower pHs; the toxic effects occur maximally between pH 5.0 and 6.0 (33). The light emission of each individual clone of the *E. coli* luciferase gene fusion library was monitored in the absence of aluminum and in the presence of aluminum at 1 and 10 μg/ml (1 and 10 ppm) at a pH of 5.5; this level of aluminum is above the background levels (approximately <0.03 to >0.67 μg/ml) found in natural waters (3). One clone, designated LF20111, was identified by its increasing gene expression (light emission) with increasing concentrations of aluminum (Fig. 1). Strain LF20111 was screened in the presence of other metals to determine the specificity of the transcriptional response as measured by light emission. Transcription was also found to increase in the presence of copper, iron, and nickel at 1 and 10 μg/ml at pH 5.5 (Fig. 1).

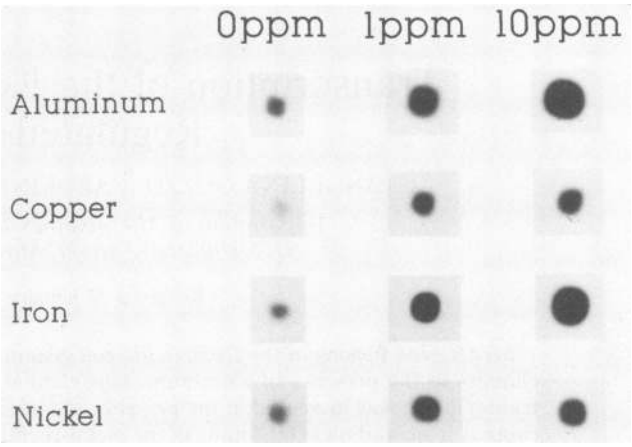

FIG. 1. Light emission of 1-cm^2 patches of strain LF20111 grown in the presence of the indicated quantities of added aluminum, copper, iron, and nickel (as $AlCl_3$, $CuSO_4$, $FeCl_3$, and $NiSO_4$) at pH 5.5. See Materials and Methods for experimental details.

Cloning and mapping of the metal-responsive gene. It was determined, by Southern blotting analysis, that the Tn*5-luxAB* element was present in the chromosome at a single location (data not shown). Thus, cloning of the right end of the gene fusion could be accomplished in one step by using the *tet* gene in the Tn*5-luxAB* element as a selectable marker. Cloning was performed by cleaving total cellular DNA from strain LF20111 with *Bam*HI and ligating the DNA to plasmid pUC119 (31) hydrolyzed with the same enzyme (Fig. 2). The ligation mixture was transformed into strain NM522 (11) with selection for tetracycline resistance. Because there are no *Bam*HI sites in *tet* and IS*50*R, the only plasmids that survive this selection should contain *tet*, IS*50*R, and the chromosomal sequences between IS*50*R and the adjacent, chromosomal *Bam*HI site (Fig. 2). The plasmid clone designated pAG5 was obtained by this procedure.

Plasmid pAG5 was digested with *Hin*dIII, and a fragment containing 1.1 kb of IS*50*R and approximately 1.2 kb of adjacent chromosomal DNA was isolated (Fig. 2). This fragment was labeled and hybridized to the ordered phage λ bank of the *E. coli* chromosome (17) and was found to hybridize to phages 341 and 342 (Fig. 3A). Thus, the junction between IS*50*R and the chromosomal DNA was contained in the area of the bacterial genome common to both phages 341 and 342.

The exact size of the chromosomal *Bam*HI fragment in which the Tn*5-luxAB* element had inserted was determined by digesting the cellular DNA of the parent strain *E. coli* DH1, in which the fusions were prepared, with *Bam*HI and hybridizing to the *Hin*dIII probe (Fig. 3C). The band was determined to be 1.8 kb in size. In strain LF20111, in which the Tn*5-luxAB* element is inserted in the chromosome, the band increases in size to approximately 8.5 kb. Another band, approximately 5 kb in size, appears that is due to cross-hybridization of the probe to the ColE1 RNA1-overproducing plasmid pTF421 (data not shown).

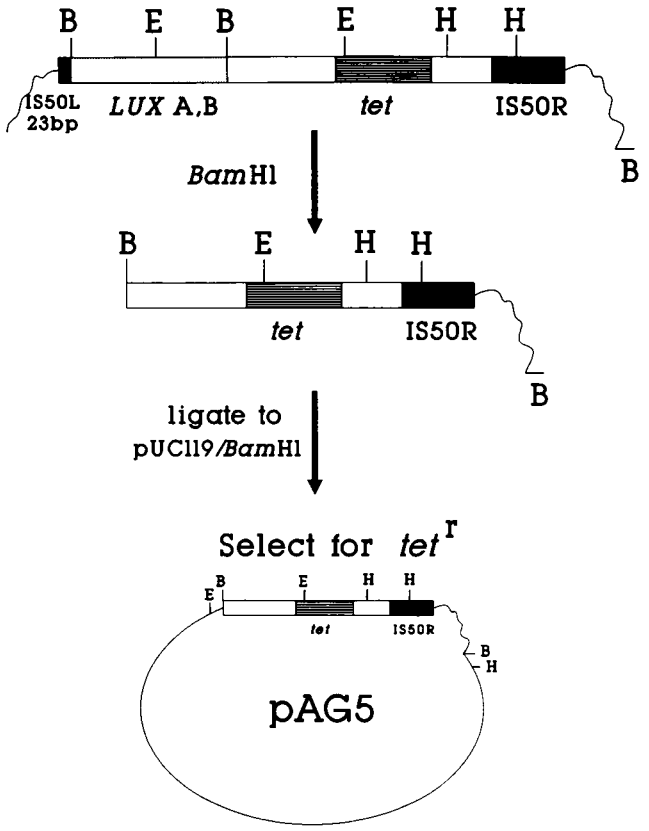

FIG. 2. Schematic diagram for the cloning of the metal-responsive gene. Restriction enzyme sites are abbreviated as follows: *Bam*HI (B), *Eco*RI (E), and *Hin*dIII (H). See Materials and Methods for experimental details.

By combining the information obtained from mapping and analysis via Southern blotting, it was determined that the Tn*5-luxAB* element had inserted approximately 1.2 kb from the first *Bam*HI site common to both phages in a right-to-left orientation on the Kohara et al. (17) restriction map of the *E. coli* genome at approximately 42.4 min, in the vicinity of *fliC* (2).

Sequencing and identification of the metal-responsive gene. To precisely identify the metal-responsive gene, the junction between IS*50*R and the site of Tn*5-luxAB* insertion was sequenced from the plasmid pAG5. The sequence obtained was analyzed by a computer homology search against the GenBank data bank; the *fliC* gene, encoding flagellin, was identified. The site of insertion is 97 bp downstream from the start of the *fliC* coding sequence (20) (Fig. 3B) and in the correct orientation for the *luxAB* genes to be under the control of the *fliC* promoter region. DNA sequencing was also performed at the other *Bam*HI junction to rule out the possibility of chromosomal rearrangements that may have occurred at the site of insertion. This was found to be the *Bam*HI site situated at bp 1224 of *fliC*, in good agreement with the distances obtained from gel electrophoresis of pAG5 restriction fragments and the Southern blot of strains DH1 and LF20111 (Fig. 3C).

DISCUSSION

Aluminum is the most abundant metal in the Earth's crust (15). It has been noted that aluminum displays increasing toxicity to living organisms as the pH is lowered below 6.0, presumably due to its increasing solubility (3, 6, 7, 15, 33). To identify any genetically programmed responses that play a role in coping with the stress of elevated levels of environmental aluminum, *luxAB* gene fusions in the *E. coli* chromosome were screened at pH 5.5 in the absence and presence of aluminum. A clone was found that had the *luxAB* genes fused to a gene whose transcription was increased in the presence of aluminum, as well as copper, nickel, and iron, at pH 5.5 (Fig. 1). Zinc was also found to induce transcription, whereas lithium had little, if any, effect (data not shown). Cloning, mapping, and sequencing revealed that the *luxAB* genes were inserted 97 bp downstream from the start codon of the *fliC* coding sequence (Fig. 3).

The *fliC* gene encodes flagellin, which is the single protein that makes up the filament of the *E. coli* flagellum (21). *fliC-lacZ* gene fusions have been previously studied to determine the effects on transcription by other proteins involved in the assembly and regulation of the flagellum (18, 19). It was found that *fliC* transcription is controlled by many steps in a complex, regulatory cascade mechanism. Any defect in a step in the regulatory cascade resulted in decreased expression of *fliC* and thus reduced synthesis of the flagellar filament (18, 19, 21). Assembly of the flagellum is costly to the cell; the growth disadvantage of synthesis relative to cells not synthesizing a flagellum was estimated to be about 2%, as measured on the basis of growth rate (21). However, the flagellum can also provide a growth advantage, since it is part of the machinery that enables *E. coli* to migrate toward nutrients and away from toxicants (22).

We have found that *fliC* transcription can be induced by the presence of low concentrations of metal ions. It is still not clear at which step, if any, in the regulatory cascade the metal ions mediate the increase in *fliC* transcription. Stimulation of *fliC* gene expression in the presence of aluminum occurred at pH 5.5 but very little occurred at pH 7.0 (data not shown). This response may be biologically relevant, since the cell should be expending energy only in the presence of aluminum at doses that would harm the cell and only at concentrations that are above the natural background levels in the environment. At pH 7.0, the toxicity of aluminum is reduced due to its decreased solubility, possibly explaining the lack of *fliC* induction at pH 7.0. If the *fliC* gene product were important to cell survival in the presence of heavy metals, the Tn*5-luxAB* insertion in the gene would disrupt it, and thus strain LF20111 would be more sensitive than the wild-type strain DH1 to elevated concentrations of aluminum. No significant differences in MIC were found when strains LF20111 and DH1 were plated on LB media containing aluminum at pH 5.5 or pH 7.0 (data not shown).

Two transcriptional regulatory proteins have been shown to require iron (Fur) or divalent metal ions (Fnr) for activity. Both the Fur and Fnr proteins have a consensus binding sequence upstream of the start of transcription (14). Neither consensus binding site was found upstream of the start site of *fliC* transcription.

Heavy metals have complex chemistries, and their concentrations in aquatic environments depend upon many factors, including pH, E°, and the presence of organic ligands (7, 33). Also, metal concentrations are not always constant. For example, in spring, melting snow can produce a strong acid and aluminum pulse (27). The ability to quickly and easily measure the bioavailability of metal ions above background levels in the environment would be useful for early detection of metal ion contamination in aqueous samples. Strain LF20111 showed increased light emission in the

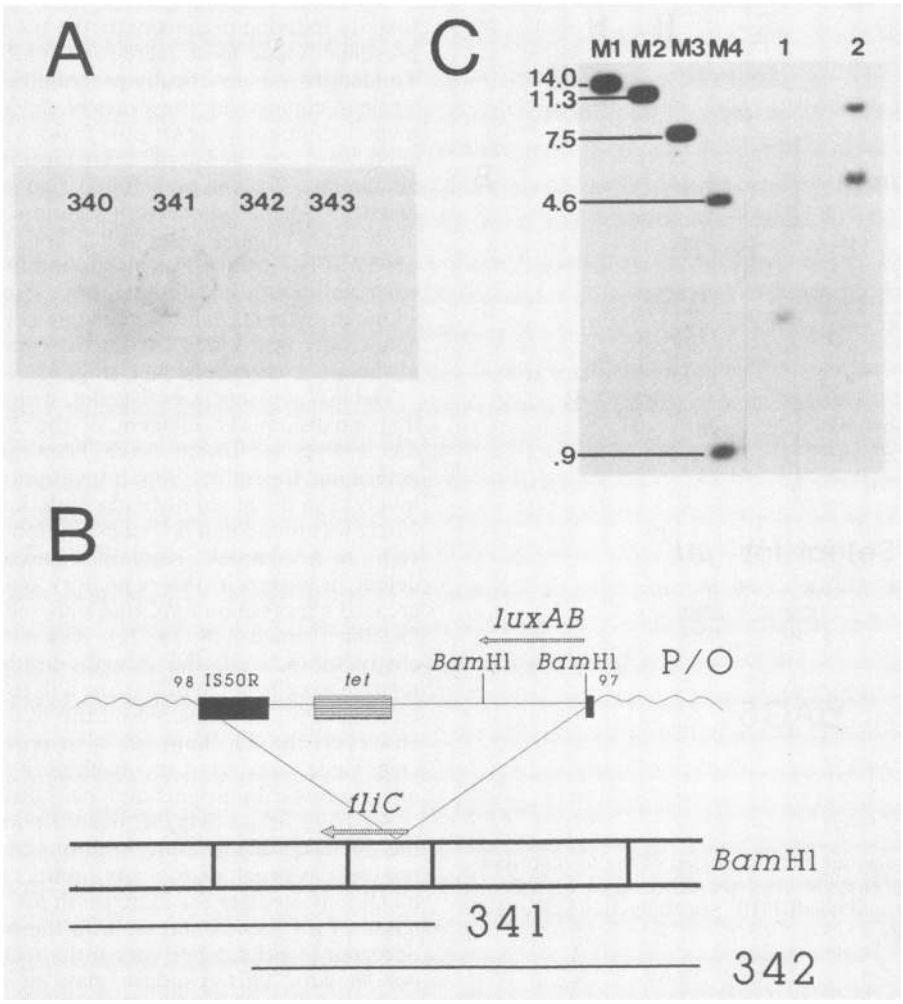

FIG. 3. Strategy for the determination of the genomic location of the metal-responsive gene. (A) Hybridization to the ordered phage λ *E. coli* gene bank (17). Shown is the hybridization to plaques containing DNA from phages 341 and 342. Plaques containing DNA from phages 340 and 343 are also shown. (B) Transcription map from the *fliC* promoter-operator through the *luxAB* genes in relation to the published *Bam*HI restriction map corresponding to phages 341 and 342 (17). Sequencing localized the site of the Tn*5*-*luxAB* insertion to between bp 97 and 98 with respect to the start of the *fliC* coding sequence (20). (C) Southern blot analysis of *Bam*HI-cleaved DNA of the parent strain DH1 (lane 1) and strain LF20111 (lane 2). Sizes in kilobases of marker DNAs (lanes M1 through M4) are indicated at the left.

presence of several metal ions. However, the *fliC*::Tn*5*-*luxAB* bacterial clone described here may prove useful as the starting material for engineering a bioluminescent biosensor to measure elevated amounts of only heavy, toxic metal ions in aquatic environments with current in vivo and in vitro genetic manipulations. The advantages of using a microorganism for detection are that the amount of metal measured is equivalent to the amount available to living organisms and the rapidity and ease of the bioluminescence assay (4). Moreover, since the reagent (strain LF20111) is living, unlimited quantities can be inexpensively prepared.

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REFERENCES

1. **Auerswald, E. A., G. Ludwig, and H. Schaller.** 1980. Structural analysis of Tn*5*. Cold Spring Harbor Symp. Quant. Biol. **45**:107–113.
2. **Bachmann, B.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54**:130–197.
3. **Birchall, J. D., C. Exley, J. S. Chapell, and M. J. Phillips.** 1989. Acute toxicity of aluminum to fish eliminated in silicon-rich acid waters. Nature (London) **338**:146–148.
4. **Bulich, A. A.** 1984. Microtox: a bacterial toxicity test with several environmental applications, p. 55–64. *In* D. Liu and B. J. Dutka (ed.), Toxicity screening procedures using bacterial systems. Marcel Dekker, Inc., New York.
5. **Collins, Y. E., and G. Stotzky.** 1989. Factors affecting the toxicity of heavy metals to microbes, p. 31–90. *In* T. J. Beveridge and R. J. Doyle (ed.), Metal ions and bacteria. John Wiley & Sons, Inc., Toronto.
6. **Cronan, C. S., and C. L. Schofield.** 1979. Aluminum leaching response to acid precipitation: effects on high-elevation water-

sheds in the Northeast. Science **204**:304–306.
7. **Cronan, C. S., W. J. Walker, and P. R. Bloom.** 1986. Predicting aqueous aluminum concentrations in natural waters. Nature (London) **324**:140–143.
8. **Engebrecht, J., M. Simon, and M. Silverman.** 1985. Measuring gene expression with light. Science **227**:1345–1347.
9. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132**:6–13.
10. **Fitzwater, T., J. Tamm, and B. Polisky.** 1984. RNA1 is sufficient to mediate plasmid ColE1 incompatibility *in vivo*. J. Mol. Biol. **175**:409–417.
11. **Gough, J., and N. Murray.** 1983. Sequence diversity among related genes for recognition of specific targets in DNA molecules. J. Mol. Biol. **166**:1–19.
12. **Guzzo, A., and M. S. DuBow.** Submitted for publication.
13. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**:557–580.
14. **Hennecke, H.** 1990. Regulation of bacterial gene expression by metal-protein complexes. Mol. Microbiol. **4**:1621–1628.
15. **Johnson, A. C., and M. Wood.** 1990. DNA, a possible site of action of aluminum in *Rhizobium* spp. Appl. Environ. Microbiol. **56**:3629–3633.
16. **Johnson, R. C., and W. S. Reznikoff.** 1983. DNA sequences at the ends of transposon Tn*5* required for transposition. Nature (London) **304**:280–282.
17. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50**:495–508.
18. **Komeda, Y.** 1982. Fusions of flagellar operons to lactose genes on a Mu *lac* bacteriophage. J. Bacteriol. **150**:16–26.
19. **Komeda, Y., and T. Iino.** 1979. Regulation of expression of the flagellin gene (*hag*) in *Escherichia coli* K-12: analysis of *hag-lac* gene fusions. J. Bacteriol. **139**:721–729.
20. **Kuwajima, G., J. Asaka, T. Fujiwara, T. Fujiwara, K. Node, and E. Kondo.** 1986. Nucleotide sequence of the *hag* gene encoding flagellin of *Escherichia coli*. J. Bacteriol. **168**:1479–1483.
21. **Macnab, R. M.** 1987. Flagella, p. 70–83. *In* F. C. Neidhardt, J. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
22. **Macnab, R. M.** 1987. Motility and chemotaxis, p. 732–759. *In* F. C. Neidhardt, J. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
23. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Miyamoto, C. M., A. D. Graham, M. Boylan, J. F. Evans, K. W. Hasel, E. A. Meighen, and A. F. Graham.** 1985. Polycistronic mRNAs code for polypeptides of the *Vibrio harveyi* luminescence system. J. Bacteriol. **161**:995–1001.
27. **Schindler, D. W.** 1988. Effects of acid rain on freshwater ecosystems. Science **239**:149–157.
28. **Silver, S., and T. K. Misra.** 1988. Plasmid-mediated heavy metal resistances. Annu. Rev. Microb. **42**:717–742.
29. **Smith, E. G., and M. D. Summers.** 1980. The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzyloxymethyl-paper. Anal. Biochem. **109**:123–129.
30. **Tolias, P. P., and M. S. DuBow.** 1987. The amino terminus of the bacteriophage D108 transposase protein contains a two-component, sequence-specific DNA-binding domain. Virology **157**: 117–126.
31. **Vieira, J., and J. Messing.** 1987. Production of single-stranded plasmid DNA. Methods Enzymol. **153**:3–11.
32. **Whittaker, P. A., A. J. B. Campbell, E. M. Southern, and N. E. Murray.** 1988. Enhanced recovery and restriction mapping of DNA fragments cloned in a new λ vector. Nucleic Acids Res. **16**:6725–6736.
33. **Wood, J. M.** 1988. Transport, bioaccumulation, and toxicity of metals and metalloids in microorganisms under environmental stress, p. 309–312. *In* J. R. Kramer and H. E. Allen (ed.), Metal speciation: theory, analysis and application. Lewis Publishers, Chelsea, Mich.
34. **Ziegler, M. M., and T. O. Baldwin.** 1981. Biochemistry of bacterial bioluminescence. Curr. Top. Bioenerg. **12**:65–113.