Excretion of Enterochelin by \textit{exbA} and \textit{exbB} Mutants of \textit{Escherichia coli}

S. K. GUTERMAN,\textsuperscript{1} AND L. DANN\textsuperscript{2}

Biology Department, Brandeis University, Waltham, Massachusetts 02154

Received for publication 18 January 1973

\textit{Escherichia coli} mutants that are insensitive to colicins B and I hyperproduce and excrete the iron chelator enterochelin, which is an inhibitor of these colicins. These mutants are classified as \textit{exbA} and \textit{exbB}. The \textit{exbA} mutants are chromium sensitive and require iron for growth, and the mutations are located in the \textit{tonB} region at min 25 of the \textit{E. coli} chromosome. \textit{tonB} mutants in which the genome of phage \textit{lambda} is inserted into the bacterial chromosome within the \textit{tonB} gene also exhibit enterochelin excretion. The \textit{exb} mutants require methionine and probably result from deletions which are located between min 56 and 58. Colicin insensitivity, enterochelin excretion and methionine auxotrophy are recessive in \textit{exbB} merodiploids. The methionine requirement of \textit{exbB} strains is satisfied by cystathionine or homocysteine, and \textit{exb} mutants are sensitive to ethionine.

Colicin B- and I-insensitive mutants were described by Gratia (6). Some of the mutations conferred resistance to phage T1, were mapped in the \textit{tonB} gene near the tryptophan operon, and were frequently deletions (7). The \textit{tonB} gene has recently been involved in iron transport: \textit{tonB} mutants are chromium sensitive (16) and show decreased binding constants for iron in kinetic studies of iron transport (17). Gratia (6) described another class of mutants insensitive to colicins B and I which required methionine. The corresponding mutation was located in the section of the \textit{E. coli} chromosome between \textit{his} at min 39 and \textit{str} at min 65.

We present evidence concerning the excretion of enterochelin by a series of newly isolated mutants insensitive to colicins B and I, as well as the physiology of these mutants and the location of the corresponding genes.

MATERIALS AND METHODS

\textbf{Bacteria and phage.} Bacterial strains are listed in Table 1 or have been described previously (8). Phage T5 and T6 were obtained from S. E. Luria; \(\phi 80\mathrm{vir}\), from E. R. Signer; \(\lambda c 857\) and \(\lambda c 60\), from R. Susman; P1 and R17, from R. Schleif. Isolation of \textit{exb} mutants from colicin-sensitive strains has been described (8).

\textbf{Media.} OM minimal medium contains per liter of distilled water: 10.5 g of K\textsubscript{2}HPO\textsubscript{4}; 4.5 g of KH\textsubscript{2}PO\textsubscript{4}; 0.05 g of MgSO\textsubscript{4}; 1.0 g of (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, and 1.6 mM sodium citrate. Other media have been described (8). Minimal media were supplemented with 0.2% glucose and 1 \(\mu\)g of thiamine per ml.

Phage P1 was titered on LB agar with 2.5 \(\times\) \(10^{-4}\) M CaCl\textsubscript{2} and 0.1% glucose. Chromium sensitivity was determined on LB agar supplemented with 4 \(\times\) \(10^{-4}\) M CrCl\textsubscript{3}.

\textbf{Colicin techniques.} Preparation and titration of colicin B and colicin inhibitor have been described (8).

\textbf{Bacterial conjugation.} Matings were performed according to Nagel de Zwaag and Luria (12). A recA derivative of strain LD60 \textit{exbB metL serA} \textit{tsx} was constructed by conjugation with Hfr 111 \textit{recA nalB} and selection for recombinants resistant to T6 and to nalidixic acid and the \textit{recA} phenotype (sensitivity to ultraviolet light or methylmethane sulfonate). Into one such recombinant, LD63 \textit{recA nalB exbB serA metL tex}, the episome \(F^+\) KLF16 containing genes from \textit{metC} to \textit{fuc} was introduced by mating with strain KL110/KLF16, and merodiploids were selected by plating on minimal agar with methionine. The exconjugants that grew were the merodiploids LD63/ KLF16.

\textbf{Transduction.} Recipient cells for P1 transductions were grown in LB broth to log phase, and CaCl\textsubscript{2} was added to 5 \(\times\) \(10^{-3}\) M. Phage P1 grown on the appropriate donor was added at a multiplicity of 0.1 to 0.2 phage per cell. After 15 min at 37 C, samples of the mixture or controls were plated on selective media.

\textbf{Selection of \(\lambda\)-induced \textit{tonB} mutants.} Log-phase cells of strain B(583) \(\Delta 24\) (\textit{gal bio hatt}) were infected with \(\lambda c 857\) (temperature inducible) and grown 4 h at...
Isolation and phenotype of exb mutants. Independent spontaneous colicin-insensitive mutants of *E. coli* K-12 strain C600 were obtained (8) by selection with colicin produced by strains C1 139 (Col B, M), CA53 (Col Ia), C600 (Col Ib), or K94 (Col V). Mutants fell into various classes: insensitive to colicin B, I, or V; or to B and I, I and V, and B and V; or to all three colicins B, I, and V. Response of mutants to colicins Ia and Ib was identical. Some of these excrete large quantities of enterochelin, an inhibitor of colicins B and I, and have been named *exb* mutants (8). Some of these mutants have a requirement for methionine.

Enterochelin production in wild-type strains is repressed by exogenous iron (3,18). In *exb* strains, enterochelin is produced in high quantities in media containing up to 2 mM FeCl₃. Of the two classes of *exb* mutants distinguished genetically below, the *exbA* type produces a larger quantity of colicin inhibitor than the *exbB* (Fig. 1). For example, GUC6 *exbA* and GUC41 *exbB* produced 1,000- and 250-fold more enterochelin, respectively, than strain C600, as judged by titration of culture supernatant fluids against colicin B (8).

**Table 1. Bacterial strains, *E. coli* K-12**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source*</th>
</tr>
</thead>
<tbody>
<tr>
<td>GUC6</td>
<td><em>exbA</em> from C600</td>
<td>this paper</td>
</tr>
<tr>
<td>GUC12</td>
<td><em>exbA</em> from C600</td>
<td>this paper</td>
</tr>
<tr>
<td>GUC41</td>
<td><em>exbB metL</em> from C600</td>
<td>this paper</td>
</tr>
<tr>
<td>GUC49</td>
<td>colicin B insensitive, from C600</td>
<td>this paper</td>
</tr>
<tr>
<td>C600</td>
<td><em>thr leu tonA</em></td>
<td>LI04</td>
</tr>
<tr>
<td>W4032</td>
<td>pro met tsx Hfr Cav</td>
<td>LA235</td>
</tr>
<tr>
<td>GUW5</td>
<td><em>exbA</em> from W4032</td>
<td>this paper</td>
</tr>
<tr>
<td>PA309</td>
<td><em>thr leu trp his arg thi str F⁻</em></td>
<td>LA435</td>
</tr>
<tr>
<td>WA5028a</td>
<td><em>trpA</em> double-point mutant</td>
<td>E. Signer</td>
</tr>
<tr>
<td>X5050</td>
<td><em>lac-pro deletion</em> (Δ90lac)</td>
<td>E. Signer</td>
</tr>
<tr>
<td>VXII</td>
<td>deletion from <em>trp</em> to <em>lac</em> in phage, from X5050</td>
<td>E. Signer</td>
</tr>
<tr>
<td>M107</td>
<td>sul</td>
<td></td>
</tr>
<tr>
<td>B(583)Δ24</td>
<td><em>gal hatt bio deletion</em></td>
<td></td>
</tr>
<tr>
<td>3LH9</td>
<td><em>tonB</em> (Δ5857) from B(583)Δ24</td>
<td></td>
</tr>
<tr>
<td>P10</td>
<td><em>thr leu malB lac str</em></td>
<td></td>
</tr>
<tr>
<td>LD28</td>
<td><em>exbB metL</em> from P10</td>
<td>this paper</td>
</tr>
<tr>
<td>A2325</td>
<td><em>argE his pro trp mtl gal tsx F⁻</em></td>
<td>D. Boyd</td>
</tr>
<tr>
<td>LD54</td>
<td><em>exbB metL</em> from A2325</td>
<td>this paper</td>
</tr>
<tr>
<td>KLI6</td>
<td>Hfr</td>
<td>LA761</td>
</tr>
<tr>
<td>AB856</td>
<td><em>serA6</em></td>
<td>B. Bachmann</td>
</tr>
<tr>
<td>LD60</td>
<td><em>serA exbB metL tsx</em> from AB856</td>
<td>this paper</td>
</tr>
<tr>
<td>LD63</td>
<td><em>serA exbB metL recA nalB tsx</em> from LD60 and Hfr 111</td>
<td>this paper</td>
</tr>
<tr>
<td>Hfr 111</td>
<td>recA nalB</td>
<td></td>
</tr>
<tr>
<td>KL110/KLF16</td>
<td>chromosome: <em>argG6 metB1 his-1 thy-23 leu-6 recA1; F⁻: from AB312, metC</em> to fuc*</td>
<td></td>
</tr>
</tbody>
</table>

* Symbols refer to Luria stock collection.

30 C. Samples were coaggregated with sufficient colicin B, ΔΦ80vir and Δλc80 so that, on control plates with any one of these three agents, most of the cells were killed. Surviving colonies were picked to duplicate grids on LB plates which were incubated at 30 or 42 C. Twelve ts isolates were obtained, three of which produced phage lambda and were considered to be *tonB* (Δ5857).

High-titer λ for transduction was prepared from one such strain 3LH9 and tested for ability to transduce *Trp*⁺ to strain WA5028a *trpA*. Transductants appeared at a frequency of 3 × 10⁻⁸.

**RESULTS**

**Isolation and phenotype of exb mutants.** Independent spontaneous colicin-insensitive
To determine if \textit{exb} mutants from C600 were 
tonB we used transduction of the \textit{trp}A gene 
adjacent to \textit{tonB}. Strain WA5028a, a double-
point mutant in \textit{trp}A, was infected with P1 
grown on GUC12 \textit{exb}A, GUC41\textit{exb}B, or C600, 
and \textit{trp}+ transductants were selected and tested 
for phage and colicin sensitivity (Table 3). 
Eighty-four percent of \textit{trp}+ transductants from 
P1-GUC12 were \textit{tonB} and \textit{exb}. All transduc-
tants from C600 and GUC41 were wild type. 
Thus strain GUC12 \textit{exb} is \textit{tonA} \textit{tonB}.

\textit{tonB} mutants require supplementary iron for 
growth in minimal medium and are inhibited by 
chromium (16, 17). Growth of strain 
GUC12\textit{exb}A was totally inhibited by CrCl,
(Fig. 2) as were the \textit{trp}A+ \textit{tonB} transductants from 
this strain. Strain GUC12 grew poorly in 
minimal medium, but grew at wild-type rates if 
supplemented with 100 \mu M FeCl,
(Fig. 3).

\textit{tonB} mutants are \textit{exb}. The above data 
indicate that certain mutants which we identify 
as \textit{exb}A are \textit{tonB}. To determine if this relation-
ship is reciprocal, \textit{tonB} mutants from other 
laboratories were tested. Strain VXII (provided 
by E. R. Signer) was prepared from X5050 by 
selection with colicin V and phage \textit{\phi}80\textit{vir} and 
contains a deletion extending from \textit{trp} to \textit{lac} in 
prophage \textit{\phi}80\textit{dlac}. Strain VXII was found to be 
colicin B insensitive and \textit{exb}. Mu-induced \textit{tonB} 
mutants (provided by M. Howe) were obtained 
from cells of strain M107 infected with phage 
\textit{mu} by selection with colicin B and \textit{\phi}80\textit{vir}. 
Phage \textit{mu} was inserted in the \textit{tonB} gene since 
\textit{trp}A+ \textit{tonB} derivatives by P1 transduction were 
lysogetic for \textit{mu}. Mu-induced \textit{tonB} as well as 
sporontaneous \textit{tonB} mutants from infected cells 
produced high levels of colicin inhibitor (Table 
4).

\textit{tonB} phenotype can be produced by inser-
tion of \textit{lambda}. Since \textit{tonB} mutants frequently 
carry deletions, the various effects of the muta-
tion might reflect a loss of different genes rather 
than pleiotropic effects of a lesion in a single 
gene. One test for pleiotropic effects was to 
isoal late chromium-resistant revertants from an 
\textit{exb}A strain and test for the other properties.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Selected markers & No. tested & Thr+Leu+ & Arg+ & His+ & Trp+ & Met+ & Pro+ \\
\hline
Thr+Leu+ & 100 & 14 & 0 & 0 & 32 & 72 & 15 \\
Arg+ & 100 & 45 & 0 & 0 & 88 & 43 & 30 \\
His+ & 98 & 15 & 19 & 14 & 17 & 22 & 56 \\
Trp+ & 98 & 14 & 8 & 16 & 5 & 16 & 88 \\
\hline
\end{tabular}
\caption{Linkage of \textit{exb}A and \textit{trp}A}
\end{table}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
Donor & \textit{Trp}+ transduc-
tsants & Sensitivity to \textit{tonB} & \textit{Col} B & \textit{\phi}80\textit{vir} & T5 & \%
\hline
C600 & 35 & + & + & + & 0 \\
GUC12 \textit{exb}A & 27 & + & + & + & 84 \\
GUC41 \textit{exb}B & 50 & + & + & + & 0 \\
\hline
\end{tabular}
\caption{Co-transduction of \textit{exb}A and \textit{trp}A}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Chromium sensitivity of an \textit{exb}A mutant. Chromium chloride was added to nutrient broth 
cultures in early log phase.}
\end{figure}

Twelve such revertants from GUC12 \textit{exb}A 
proved to be colicin insensitive, iron requiring, 
and \textit{exb}, and yielded chromium-sensitive \textit{tonB} 
strains by transduction of \textit{trp}A+ to WA5028a. In 
these revertants, as possibly also in other \textit{tonB} 
revertants selected for chromium resistance, the 
resistance may have arisen by extragenic sup-
pression.

To test in another way if all the \textit{tonB} effects 
can result from disfunction within a single 
cistron, \textit{lambda}-induced \textit{tonB} mutants were 
obtained in a strain of \textit{E. coli} K-12 with a
deletion of the normal attachment site. Shimada et al. (13) demonstrated that infection of a strain lacking the normal lambda attachment site yielded some lysogens in which the prophage had become inserted inside bacterial genes inactivating them. Reversion to wild type results in simultaneous loss of the prophage, indicating that insertion and excision of lambda occur with exact precision. Lambda-induced tonB mutants were obtained in strain B(583) Δ24(gal bio λatt) by infection with λC857 (temperature inducible) and selection with colicin B, φ80vir and λC600. Three temperature-sensitive phage-producing strains were obtained. High-titer lambda, prepared from strain 3LH9, mediated specialized trp transduction, indicating that the prophage was inserted next to the trpA gene. These lambda-induced tonB mutants were insensitive to colicins B, 1a, and 1b, required iron for growth in minimal medium, and were chromium sensitive and exb (Table 4). These phenotypes therefore can result from a mutation in a single cistron, assuming that the insertion of lambda does not result in polarity effects.

**Genetic analysis of exbB mutants.** Exb Met+ strains are T1 sensitive, chromium resistant, and grow on minimal medium without supplementary iron. The ExbB phenotype is probably due to a deletion since no Met+ revertant colonies could be obtained by plating exbB cells on medium lacking methionine.

To locate the exbB gene on the E. coli chromosome, strain LD28 (an exbB mutant of Hfr strain P10 which inserts from min 79 in the order arg, mtl, his) was crossed with A2325 F- argE his mtl; Arg+ and Mtl+ recombinants were selected at specific time intervals. The colicin-insensitive phenotype appeared between 30 and 40 min after mixing the strains, and was more closely linked to mtl than to arg. Correction for a lag in entry of markers located the exbB gene between 49 and 59 min. Conjugation of strain LD54 F- his exbB met with Hfr KL16, which inserts from min 55 with his+, an early marker, yielded his+ recombinants that were exbB like the F- parent. These data suggest that exbB is located between min 55 and 59.

Transduction of the exbB gene by phage P1 was performed to determine linkage to serA at min 57. Nine percent of serA+ transductants (6 out of 66) with P1 grown on an exbB donor acquired colicin insensitivity and methionine.

**Table 4. Production of colicin inhibitor by mu-induced or lambda-induced tonB mutants**

<table>
<thead>
<tr>
<th>Supernatant fluid*</th>
<th>Inhibition of colicin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tonB (mu)</td>
<td>96</td>
</tr>
<tr>
<td>ara-20 (mu)</td>
<td>16</td>
</tr>
<tr>
<td>tonB spontaneous</td>
<td>97</td>
</tr>
<tr>
<td>M107, parent</td>
<td>13</td>
</tr>
<tr>
<td>3LH4 tonB (lambda)</td>
<td>97</td>
</tr>
<tr>
<td>3LH5 tonB (lambda)</td>
<td>99</td>
</tr>
<tr>
<td>3LH9 tonB (lambda)</td>
<td>99</td>
</tr>
<tr>
<td>B(583)Δ24, parent</td>
<td>13</td>
</tr>
</tbody>
</table>

* Mu lysogens and controls were grown at 32°C in nutrient broth, and lambda lysogens and controls were grown at 30°C in M9 medium with 200 μM FeCl₃ and 1 μg of biotin per ml. Supernatant fluids were diluted 1:10 and titered for ability to inhibit colicin B.

**Fig. 3. Iron requirement of an exbA mutant.** A, Strains were grown in M9 medium supplemented with FeCl₃ as indicated. B, Strain GUC12, an exbA mutant from C600, was grown in M9 medium with 0, 1, 10, or 100 μM FeCl₃ added. Arrows indicate values corrected for pink color of the culture fluid.
auxotrophy. These transductants hyperexcreted colicin inhibitor in amounts comparable to the donor strain (Table 5). Thus, the exbB gene is located within about 1 min of serA.

The exbB phenotype is recessive. Strain LD63 recA tsx exbB met serA was mated with KL110/KLF16, which contains the F' episome F16 including genes from metC at min 59 to fuc at min 54. Presumed merodiploid recombinants were selected on minimal medium supplemented only with methionine. These recombinants were sensitive to colicin B, produced 100 times less colicin inhibitor than the exbB parent, and approximately 3 times more than the wild type as shown in Table 6, and did not require methionine.

Characteristics of the methionine auxotrophy of exbB mutants. exbB strain LD28 required at least 10 µg of methionine per ml of minimal medium for optimal growth. The methionine requirement of strain GUC41 was compared to those of a metA and a metE mutant. GUC41 grew well with either homocysteine or cystathionine, suggesting that the auxotrophy is probably not due to a defect of conversion of cystathionine to methionine.

<table>
<thead>
<tr>
<th>Supernatant fluid</th>
<th>Phenotype</th>
<th>Inhibition of colicin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transductant-1</td>
<td>Col'/Met'</td>
<td>96</td>
</tr>
<tr>
<td>Transductant-2</td>
<td>Col'/Met'</td>
<td>97</td>
</tr>
<tr>
<td>Transductant-3</td>
<td>Col'/Met'</td>
<td>7</td>
</tr>
<tr>
<td>AB856 serA (recipient)</td>
<td>Col'/Met'</td>
<td>0.5</td>
</tr>
<tr>
<td>LD28 exbB (donor)</td>
<td>Col'/Met'</td>
<td>99</td>
</tr>
</tbody>
</table>

*Phage P1 grown on strain LD28 exbB was used to transduce SerA* to strain AB856 serA. Transductants were tested for colicin sensitivity (Col' or Col*), for methionine requirement and for ability to excrete colicin inhibitor.

<table>
<thead>
<tr>
<th>Supernatant fluid</th>
<th>Inhibition of colicin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Undilated</td>
</tr>
<tr>
<td>Merodiploid-1</td>
<td>85</td>
</tr>
<tr>
<td>Merodiploid-2</td>
<td>83</td>
</tr>
<tr>
<td>Merodiploid-4</td>
<td>89</td>
</tr>
<tr>
<td>LD63 exbB parent</td>
<td>62</td>
</tr>
<tr>
<td>KL110/KLF16 parent</td>
<td>85</td>
</tr>
</tbody>
</table>

*Merodiploids were isolated from a mating of LD63 serA exbB metL recA nalB tsx with KL110/KLF16 F' episome from min 54 to 59. Merodiploids were tested for production of colicin inhibitor in M9 medium with 200 µM FeCl₃ and 20 µg of methionine per ml.

Strains of E. coli and S. typhimurium known as metK mutants are characterized by loss of sensitivity to methionine analogs including ethionine and norleucine (9). The exbB mutant LD28 and its parent P10 were tested in minimal medium containing 5 mg of ethionine and 1 µg of methionine per ml, and were fully sensitive to ethionine (Fig. 4). Analogous experiments with norleucine were inconclusive since the sensitive control strain grew at all concentrations in the presence of methionine.

### DISCUSSION

In this paper we have presented evidence concerning two types of mutations which have been mapped in different regions of the E. coli chromosome and cause excretion of large quantities of enterochelin in the presence of sufficient iron to repress synthesis of this compound in wild-type cells. exbA mutants excrete 1,000 times more enterochelin than wild-type strains, and the corresponding mutations are located in

![Fig. 4. Ethionine sensitivity of an exbB mutant. Cultures were diluted into M9 medium with or without methionine (1 µg per ml) and ethionine (5 mg per ml). A, Parent strain P10. B, Strain LD28exbB.](http://jb.asm.org)
the *tonB* gene, and *tonB* mutants isolated independently are *exb*. The *exbB* mutations that cause a 250-fold increase in enterochelin production are located between min 56 and 58, may be mainly deletions, and are recessive with respect both to colicin sensitivity and enterochelin excretion. Neither *exb* trait could result from an operator constitutive mutation since these mutations are not linked to the *ent* genes for enterochelin synthesis, which map at min 14 (5, 10).

The *tonB* gene is involved in iron uptake, hence enterochelin excretion by *exbA* mutants may be related to the internal iron pools of these cells. Bryce and Brot (3) have calculated that synthesis of 2,3-dihydroxybenzoylserine occurs in cells when the intracellular iron level falls below $22 \times 10^{-10}$ mol per cell. Assuming an *E. coli* cellular volume of $1 \mu m^3$, the internal iron level necessary to repress synthesis is 2.2 mM. It is possible that *exbA* strains cannot accumulate enough iron to repress enterochelin biosynthesis and are therefore acting as constitutive. Excretion by *exbB* mutants, which are not defective in iron transport, may be due to failure to synthesize an iron-sensitive aporepressor of the enterochelin operon.

The methionine lesion of *exbB* mutants is satisfied by homocysteine and cystathionine. This observation and the map position rule out a mutation in known methionine biosynthetic enzymes or a defect in *metG* which maps near the histidine operon in *Salmonella typhimurium* (14) and probably at a comparable position in *E. coli* (1). Mutants insensitive to the methionine analog ethionine, known as *metK*, map at min 57 (11). The sensitivity of *exbB* strains to ethionine rules out the identity of *exbB* with *metK*. One possible basis of the *exbB* methionine auxotrophy is that these mutants lack a positive controlling substance for transcription or translation of one or more of the genes involved in methionine biosynthesis. The methionine auxotrophy associated with the mutation may be called *metL*.

The *tonB* region is one of the classical deletion systems in *E. coli* (4), and the exact relationship of fine structure to function has remained obscure. Our data on the lambda-containing *tonB* strains indicate that a defect in a single cistron can cause loss of all the properties attributed to the *tonB* gene, assuming no polarity effects from the inserted prophage. Gratia (6) found that different combinations of T1 and the colicin sensitivity mapped in this region and that these phenotypes are recessive. The recessive character indicates that an operator defect cannot be invoked to explain the pleiotropic effects of *tonB*. Further work is needed to clarify the relationship of the *tonB* gene product to the mutant phenotypes.

**ACKNOWLEDGMENTS**

We thank D. Boyd and R. Schleif for suggestions, S. E. Luria for discussions and criticism of the manuscript, and C. L. Howitt for expert technical assistance. This investigation was initiated while one of us (S. K. G.) held NIH predoctoral fellowship 5-F1-GM-34, 383, and was further supported by NIH Biomedical Sciences Support Grant from Brandeis University (RH07044).

**LITERATURE CITED**