APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 2005, p. 5614–5618 Vol. 71, No. 9
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.9.5614–5618.2005

Improved System for Construction and Analysis of Single-Copy β-Galactosidase Operon Fusions in *Yersinia enterocolitica*

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Received 22 January 2005/Accepted 21 April 2005

We report a significantly improved system for studying single-copy *lacZ* operon fusions in *Yersinia enterocolitica*: a simple procedure for the stable integration of *lacZ* operon fusions into the *ara* locus and a strain with a deletion mutation that abolishes the low level of endogenous β-galactosidase activity.

Yersinia enterocolitica is a cause of foodborne human gastroenteritis and a favored model organism for studying bacterial virulence (2). *Y. enterocolitica* virulence gene regulation is studied in many laboratories. This is especially true for the genes encoding the Ysc type III secretion system, which *Y. enterocolitica* shares in common with the other pathogenic *Yersinia* species, *Y. pestis* and *Y. pseudotuberculosis* (5).

In *Escherichia coli* K-12, the ability to construct single-copy *lacZ* operon fusions greatly facilitates regulatory studies. Single-copy *lacZ* fusions overcome the problems of multicopy fusions, such as copy number variation and titration of transcription factors. The only existing method for constructing single-copy *lacZ* fusions in *Y. enterocolitica* relies on complete chromosomal integration of a suicide plasmid by homologous recombination (see, e.g., reference 6). This approach has many limitations, including the following. (i) The integrated plasmid encodes an antibiotic resistance no longer available for subsequent manipulations. (ii) Chromosomal integration is driven by the promoter fragment, which limits the minimum size of the fragment that can be studied. (iii) Studying promoters internal to an operon results in disruption of the operon after suicide plasmid integration. (iv) Integration of fragments with promoter mutations may be tedious to verify (homologous recombination can occur either upstream or downstream of the mutation, and only the former places the mutation upstream of *lacZ*). The use of *lacZ* fusions in *Y. enterocolitica* is further compromised because, although it is known as a Lac$^-$ species, there is a trace level of endogenous β-galactosidase activity that causes the formation of pale blue colonies on agar containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). This limits the use of X-Gal media for phenotypic screens and the analysis of weakly expressed promoters in enzyme assays. These limitations motivated our development of an improved system for the construction and analysis of single-copy *lacZ* operon fusions in *Y. enterocolitica*.

Construction of *ara* integration plasmid pAJD905. We followed a strategy similar to that employed by Wu et al., who targeted the *rha* locus of *Klebsiella oxytoca* for *lacZ* operon fusion integration (21). They used a suicide plasmid with an *rpsL*$^+$ (streptomycin sensitivity) allele for counter selection, which requires the use of a strain with an *rpsL* mutation conferring streptomycin resistance. Streptomycin resistance is a widely used marker in *Y. enterocolitica*, which is naturally ampicillin resistant. Therefore, we chose a different approach, based on the established technology of a suicide plasmid with a π protein-dependent R6K replication origin and the use of the *sacB* gene for selection of plasmid-free segregants as sucrose-resistant colonies. We extended this technology to develop a *Y. enterocolitica* site-specific integration *lacZY* operon fusion vector, with several features that increase its utility.

For the chromosomal integration site we chose the *araFGHC-araBA* locus of a *Y. enterocolitica* strain 8081 derivative, which is the highly pathogenic strain used in the genome sequencing project. Disruption of this locus abolishes arabinose catabolism, which allows integrants to be easily identified by their Ara$^-$ phenotype (e.g., formation of white colonies on MacConkey-arabinose agar plates). It also protects the *lacZY* operon fusions from flanking promoters by placing them between divergently transcribed operons which have had their promoters deleted (10).

The allelic exchange vector pRE112 (Table 1) has a R6K replication origin and the *sacB1* gene, which confers sucrose sensitivity (9). Two 1.5-kb fragments (*'araGH'* and *'araBA'*) were amplified by PCR and ligated into the pRE112 polylinker with a unique NotI site between them (pAJD898). Next, plasmid pAJD768 was constructed by joining the ~7-kb BamHI *lacZYA-cat* fragment of pFUSE to the ~3.1-kb BglII pSC101 *ori* fragment of pWSK29 and then inserting the StuI-SmaI pSL1180 polylinker upstream of *lacZ*. Finally, the polylinker-*lacZY* region of pAJD768 was amplified with primers that incorporate *E. coli rrnBT1* and *rrnBT2* terminators upstream and downstream, respectively. This fragment was cloned into the NotI site of plasmid pAJD898. Derivatives with the *lacZY* operon in either orientation were isolated. However, one of these orientations produced somewhat higher basal *lacZY* expression than the other (data not shown). Therefore, a plasmid with the *lacZY* operon in the orientation that gave the lowest basal *lacZY* expression was chosen. This was pAJD905, the *ara*-integration *lacZY* operon fusion vector (Fig. 1A). The *rrnB* terminators further insulate *lacZY* fusions from the influence of external chromosomal regions.

Chromosomal integration of *lacZY* operon fusions. The method used to exchange *lacZY* operon fusions from pAJD905

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype or feature(s)	Reference or source
Y. enterocolitica strain 8081 derivatives		
JB580v	Δ*yenR* (R^- M^+)	12
AJD935	Δ*yenR* (R^- M^+) Δ*araGFB*::[Φ(−*lacZY*)]	This study
AJD957	Δ*yenR* (R^- M^+) Δ*araGFB*::[Φ(*pspA*−*lacZY*)]	This study
AJD1022	Δ*yenR* (R^- M^+) Δ*araGFB*::[Φ(*yopH*−*lacZY*)]	This study
AJD1024	Δ*yenR* (R^- M^+) ΔYE2592	This study
AJD1025	Δ*yenR* (R^- M^+) ΔYE2592 Δ*araGFB*::[Φ(−*lacZY*)]	This study
Y. enterocolitica CDC[a] reference strains and clinical isolates		
634-83	Serogroup O:4,32, American strain	17
637-83	Serogroup O:5,27, non-American strain	17
MC8	Bio. 1, serogroup O:9, non-American strain	17
MC17	Bio. 1, serogroup O:3, non-American strain	17
Plasmids		
pFUSE	Cm^r, *mob*$^+$ (RP4), *lacZYA*$^+$, R6K *ori*	1
pRE112	Cm^r, *mob*$^+$ (RP4), *sacB1*$^+$, R6K *ori*	9
pSL1180	Ap^r, super polylinker, *colE1 ori*	Amersham
pSR47S	Km^r, *mob*$^+$ (RP4), *sacB*$^+$, R6K *ori*	16
pVLT33	Km^r, *tacp* expression vector, RSF1010 *ori*	7
pWSK29	Ap^r, pSC101 *ori*	20
pAJD509	*tacp-yscC*$^+$ in pVLT33	This study
pAJD554	*tacp-ysaC*$^+$ in pVLT33	This study
pAJD768	Cm^r, *lacZYA*$^+$, *pSC101 ori*	This study
pAJD898	pRE112 with *′araHG′*-NotI-*′araBA′* insert	This study
pAJD905	pAJD898 with polylinker and *lacZY* from pAJD768	This study
pAJD930	pAJD905 with *pspAp* fragment	This study
pAJD952	pAJD905 with *yopHp* fragment	This study
pAJD990	pSR47S with ΔYE2592 insert	This study

[a] CDC, Centers for Disease Control and Prevention.

into the *ara* locus is summarized in Fig. 1B. First, pAJD905 (or a derivative with a promoter insert) was transferred from *E. coli* S17-1 λ*pir* (18) to *Y. enterocolitica* by conjugation. Plasmid integrants were identified by their Cm^r Ara^- phenotype on MacConkey-arabinose agar (the 1.5-kb regions flanking *lacZY* ensure that most integrations occur at *ara*). Segregants then lost the integrated plasmid by homologous recombination between the tandemly duplicated *ara* sequences. They were selected by streaking integrants on LB agar containing 10% sucrose (loss of the plasmid *sacB1* gene restores a sucrose-resistant phenotype). The sucrose-resistant colonies were replica plated onto MacConkey-arabinose agar, which identified both Ara^+ and Ara^- segregants as expected. The authenticity of the desired Ara^- segregants was confirmed by their Cm^s phenotype. Finally, the structure of segregants was confirmed by colony PCR analysis using an Expand PCR system (Roche; see Fig. 1C).

Application of the pAJD905 system. We tested the new *lacZY* fusion system with the *pspA* promoter (studied in our laboratory) and the *yopH* promoter (studied in various laboratories). Approximately 500-bp control region fragments were amplified by PCR, confirmed by DNA sequencing, and ligated between the XbaI and BglII sites of pAJD905. The Φ(*pspA-lacZ*) and Φ(*yopH-lacZ*) fusions were integrated into the *ara* locus as described above (data not shown). In both cases, the initial integration step occurred in the *ara* locus rather than the *yopH* or *pspA* locus in over 99% of cases (initially identified by an Ara^- phenotype). We have also done other studies with an approximately 2.2-kb fragment cloned into pAJD905. In this case the frequency of integration into the *ara* locus did decrease somewhat. However, the majority of integrations were still in the *ara* locus (data not shown).

Previous studies have shown that *pspA* promoter expression is induced by the YscC and YsaC secretins, with YsaC being the most potent (6, 11, 15). The Δ*araGFB*::[Φ(*pspA-lacZY*)] fusion demonstrated exactly the same pattern of regulation (Fig. 2A). The virulence plasmid (pYV)-encoded *yopH* gene is induced by a low calcium concentration at 37°C, and its expression requires the VirF transcription activator (4, 13). Consistent with this, expression of the Δ*araGFB*::[Φ(*yopH-lacZY*)] fusion was induced approximately 30-fold by these growth conditions (Fig. 2B). Furthermore, in a strain lacking pYV (and, therefore, VirF), Φ(*yopH-lacZY*) expression was essentially abolished (7 Miller units; data not shown).

These results demonstrate the functionality of the new *lacZY* fusion system. The ability to construct stable *lacZY* fusions with pYV-encoded promoters is particularly important. The alternative approach of integrating suicide plasmids into the relatively small pYV could affect the stability or copy number of pYV or the expression of pYV-encoded genes.

Elimination of *Y. enterocolitica* endogenous β-galactosidase activity. *Y. enterocolitica* is phenotypically Lac^- on MacConkey-lactose agar but has a low level of β-galactosidase activity in enzyme assays and forms blue colonies on X-Gal media. The *Y. enterocolitica* genome encodes a protein (YE2592) approximately 60% identical to *E. coli* LacZ (data not shown). To

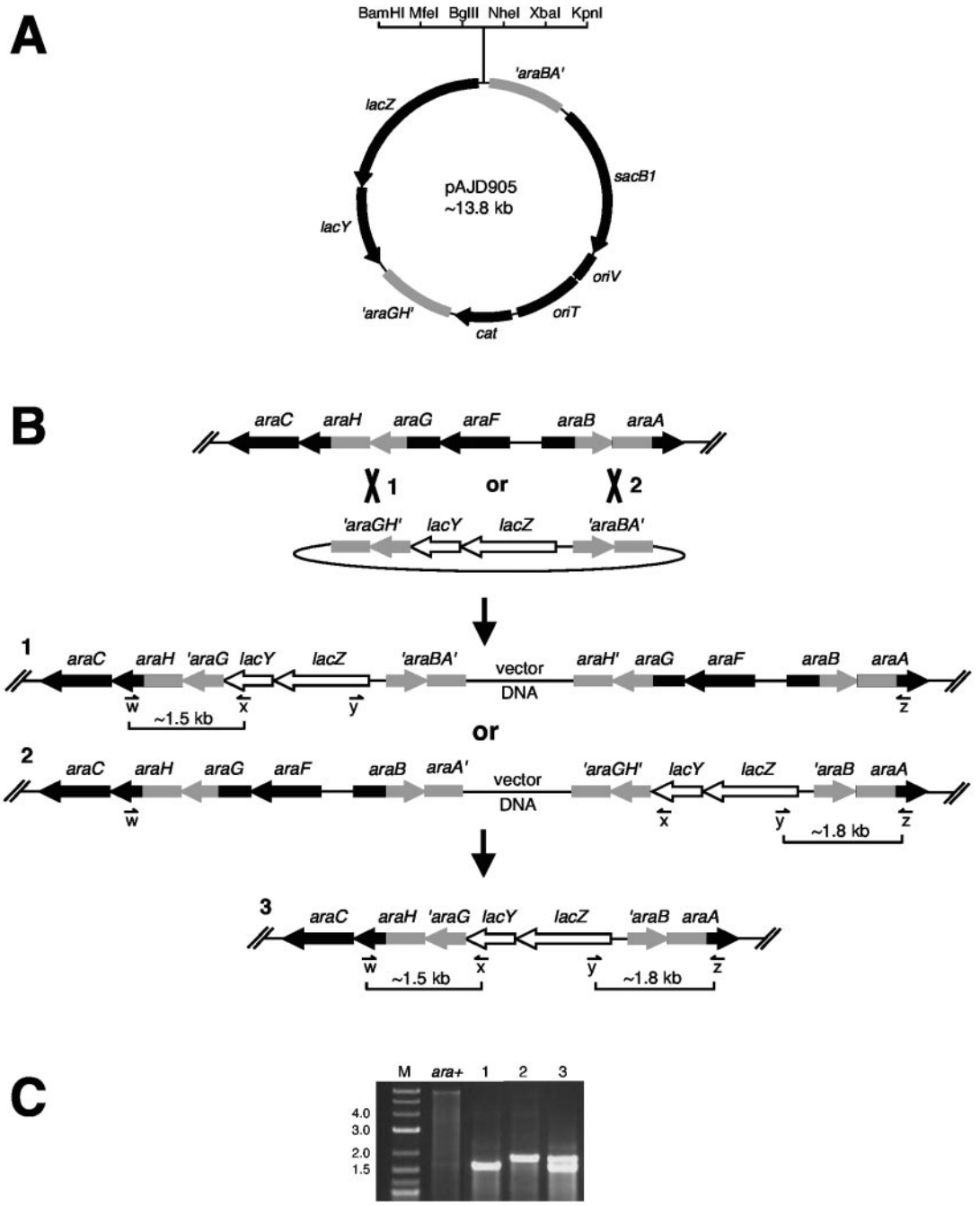

FIG. 1. Integration of *lacZY* operon fusions into the *ara* locus. (A) Partial restriction map of plasmid pAJD905. Restriction sites shown in the polylinker are unique (note that MfeI is compatible with EcoRI). (B) Summary of *lacZY* fusion integration procedure (see text for experimental details). Plasmid pAJD905 is integrated into the *ara* locus by homologous recombination between either the *'araGH'* sequences or the *'araBA'* sequences. The structures of both possible integrants are shown as structures 1 and 2. Next, sucrose-resistant segregants lose the integrated pAJD905 backbone by homologous recombination between the tandemly duplicated *ara* sequences. This either regenerates the *ara*$^+$ locus or leaves the *lacZY* fusion on the chromosome, shown as structure 3. (C) Colony PCR analysis of the *ara*$^+$ parental strain, integrants with structure 1 or 2, and segregant 3 is shown. In this case the *lacZY* operon from pAJD905 without a promoter cloned upstream was used. Binding sites of each primer are shown in panel B as w, x, y, and z (see also Table 2). For the *ara*$^+$ strain, primers w and z generate a single product of ~6 kb that is only weakly amplified (primers x and y do not anneal). Integrants 1 and 2 give only one PCR product, as indicated in panel B (the second pair of primers are too far apart for amplification). The desired segregant gives two PCR products as shown. Note that a promoter fragment inserted upstream of the *lacZY* operon would increase the size of the 1.8-kb product. M, DNA marker (kilobase sizes of some marker bands are indicated). The gel lane images have been rearranged from the original order, but the data are from the same gel and a single experiment.

TABLE 2. Primers

Primer name and function	Sequence (5′ to 3′)
For amplification of *'araGH'* and *'araBA'* fragments	
'araG NotI	GAAGCGGCCGCGGGAAATCGAGCAACTGTTCCG
araH' SpeI	CGGACTAGTGGTTATCAGGGCATTTATCTTCAGC
'araB NotI	GAAGCGGCCGCTGAACGCCAGGTTGGTCTG
araA' SacI	CGGGAGCTCCTGTTTGGCTGCCGCAACG
For amplification of *lacZY* fragment with flanking *rrnB* terminators	
lacZ Up *rrnB*T1	GAACGGCCGATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATCGGTACCTCTAGAACTATAGC
lacY Dwn *rrnB*T2	AGAATGCGGCCGCAAAAAGGCCATCCGTCAGGATGGCCTTCTGCCGACATTGATTGCTTAAGCG
For colony PCR confirmation of *lacZY* operon fusion integrants	
araH check (w)	GAAATGATGTAAGCCAGACC
lacY check (x)	CGTCAGGTGAATGAAGTCGC
M13–40 (y)	GTTTTCCCAGTCACGAC
araA check (z)	GCATATTATCGCCGAAACGG
For amplification of *pspA* and *yopH* control regions	
pspAp Up	AGGCGATGGGCTATCAGCTC
pspAp Dwn	GGATCCGCACTAACTTCTGTGGAT
yopHp Up	GGCTCTAGATATTCGTTGCTATCACTGG
yopHp Dwn	GCGAGATCTAAGTTCATGCTTCCCTCC
For amplification of fragments flanking YE2592	
ΔYE2592 Up Fwd	CGCGTCGACCCCATTTAGCGCCATCTTG
ΔYE2592 Up Rev	CCGGGATCCCTGCGGTTTAACTTCTTGC
ΔYE2592 Dwn Fwd	CCGGGATCCTGGCTAAATATCGATGGT
ΔYE2592 Dwn Rev	GAAGCGGCCGCTACAGGGAGCTTCACCA

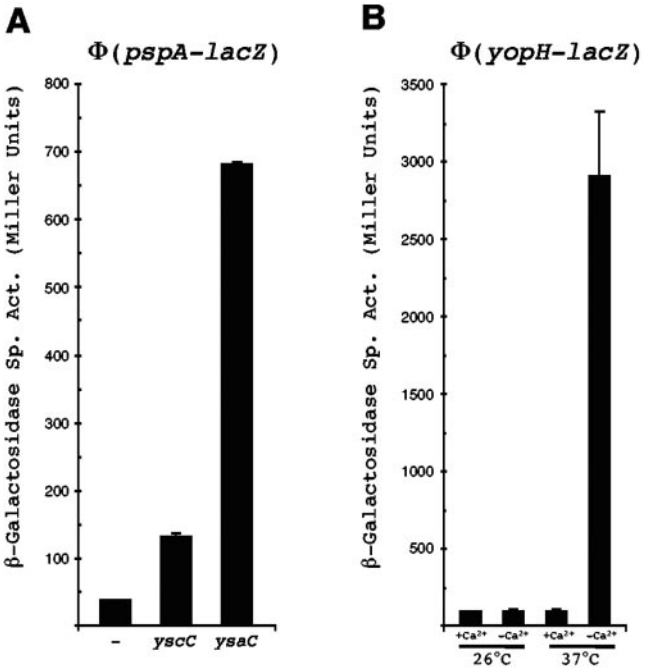

FIG. 2. Regulation of Δ*araGFB*::[Φ(*pspA-lacZY*)] and Δ*araGFB*::[Φ(*yopH-lacZY*)] fusions. (A) Φ(*pspA-lacZY*) expression. Plasmid pVLT33 (−), pAJD509 (*yscC*), or pAJD554 (*ysaC*) encoding the indicated genes expressed from the *tac* promoter were transferred into a pYV-negative derivative of strain AJD957. Strains were grown exactly as described previously (11). (B) Φ(*yopH-lacZ*) expression. Strain AJD1022 was grown at 26°C or 37°C in low- or high-calcium media as described previously (6). β-Galactosidase activities were determined as described previously (14) and are expressed in Miller units (each value is the average of measurements from three independent cultures). Sp. Act. = specific activity.

determine whether this gene is responsible for endogenous β-galactosidase activity we constructed a strain with a YE2592 in-frame deletion mutation. The mutation was made using the *sacB*$^+$ suicide plasmid pSR47S with a ΔYE2592 insert, which was generated by PCR. Plasmid construction and mutagenesis procedures were identical to those described by others (19).

In enzyme assays, the YE2592$^+$ *Y. enterocolitica* strain produced 2 to 4 Miller units of β-galactosidase activity, depending on growth conditions, whereas the ΔYE2592 mutant had no detectable activity (data not shown). Furthermore, whereas the YE2592$^+$ strain was blue on agar containing X-Gal, the ΔYE2592 mutant was white (data not shown). These results confirm that YE2592 is responsible for *Y. enterocolitica* endogenous β-galactosidase activity. The ΔYE2592 mutant strain provides a significant enhancement for the use of β-galactosidase enzyme assays and X-Gal media in screens and to analyze weakly expressed *lacZ* operon fusions.

Use of the pAJD905 system with different *Y. enterocolitica* strains. There are different biotypes and serotypes of *Y. enterocolitica* (2). In particular, there are two groups of strains known as American and non-American, which differ in their virulence properties (American strains are more virulent) (3). We were interested to know whether plasmid pAJD905 could be used in different *Y. enterocolitica* strains, in addition to our strain 8081 derivative (an American strain of serotype O:8). We randomly chose one American strain (serotype O:4,32) and three non-American strains (serotypes O:5,27; O:3; and O:9), all of which are Ara$^+$ (as were all of the *Y. enterocolitica* strains in our collection). First, we attempted PCR amplification of the *'araBA'* and *'araGH'* fragments with the same primers used to construct pAJD898. Both fragments were successfully amplified from the O:4,32; O:5,27; and O:3 strains (data not shown). However, only the *'araGH'* fragment could be amplified from the O:9 strain (therefore, this strain was not used further). Next, we attempted the pAJD905 integration and segregation procedure. For the American O:4,32 strain, the process proceeded with speed and efficiency similar to that seen with our strain 8081 derivative. The procedure was also successful for the non-American O:5,27 and O:3 strains. However, in these cases the segregation procedure heavily favored reversion to Ara$^+$. Only a few desired Ara$^-$ segregants were isolated. Even so, colony PCR analysis identified segregants

with the correct integration in both cases (data not shown). From these experiments we conclude that pAJD905 can be used in different *Y. enterocolitica* strains, although not all. However, the system is likely to be most easily used in the highly pathogenic American strains.

Summary. The *Y. enterocolitica* single-copy *lacZY* operon fusion system described here provides many benefits, including the following. (i) Fusion strains are stable, are not marked with an antibiotic resistance, and do not contain any suicide vector DNA, all of which facilitate further manipulation. (ii) There is no lower limit to the size of promoter fragments that can be analyzed. (iii) There is low background (integration of *lacZY* without a promoter fragment upstream causes only approximately 2 to 4 Miller units of β-galactosidase activity; data not shown). (iv) The native locus of the promoter under study is not disrupted (polar effects are not a concern). (v) The integration procedure is simple and takes only a few days. (vi) Correct *lacZY* fusion integrants are easily and unambiguously confirmed by their Ara^- phenotype and colony PCR analysis. Our identification of the *Y. enterocolitica* gene responsible for endogenous β-galactosidase activity, and construction of a deletion mutant, further improves the utility of *lacZ* fusions in this organism.

We thank Joe Vogel and Kimberly Walker for providing plasmid pSR47S and its DNA sequence. *Y. enterocolitica* genome sequence data were produced by the *Y. enterocolitica* Sequencing Group at the Sanger Institute and can be obtained online at http://www.sanger.ac.uk/Projects/Y_enterocolitica/.

This work was supported by Public Health Service grant AI-052148 from the National Institute of Allergy and Infectious Diseases and by a grant from the Speaker's Fund for Biomedical Research: Toward the Science of Patient Care, awarded by the City of New York.

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