Repair of promoter sequences in the transcribed strand showed a striking pattern (Fig. 2). Two slowly repaired regions (50% repair attained in 10 to 16 hours), at nucleotides −34 to −47 and at −187 to −193, occurred on the transcription-proximal side of two transcription factor binding sites, one for SP1 and the other for an unknown factor (*10*). An intervening region (nucleotides −59 to −86) was repaired at a very fast rate. The mechanism underlying the transcription factor effect is unknown. In this instance, repair is strand-specific for a nontranscribed region and is probably coupled to transcription; as such, this effect confuses historical repair nomenclature for active genes.

The frequency of UV-induced mutations at any nucleotide position depends both on damage frequency and repair rate (*11*). The frequency of CPDs was already known to vary considerably along genes (*3, 6*), and we have shown here that CPD repair rates vary 15-fold along the *PGK1* gene. Recently, hotspots for UV-induced mutations were reported to be positions of slow CPD repair in the bacterial *lacI* gene (*12*) and the human *p53* gene (*13*). Mutation frequency can now be understood by its component mutagenesis mechanisms because the final link (repair rate) is now amenable to mapping.

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4. Ligation-mediated PCR is a single-sided PCR method (*14, 15*). A set of three oligonucleotide primers are synthesized that are complementary to three overlapping sites within a sequenced region of DNA. Genomic DNA is nicked to generate broken molecules with terminal 5′-phosphate groups. After denaturation, primer 1 is annealed and extended with Sequenase to the previously induced nicks, thereby generating blunt ligatable ends. Ligation of a double-stranded oligonucleotide linker onto the 5′ end of each DNA molecule provides a common sequence at all previously broken ends. Primer 2, whose sequence partially overlaps that of primer 1, is then used in conjunction with the common linker sequence in a PCR reaction. After 19 to 21 PCR cycles, the amplified DNA fragments are separated on a sequencing gel, electroblotted onto a nylon membrane, and hybridized to a single-stranded, gene-specific ^{32}P-labeled probe [synthesized by PCR with primer 3 (*16*)]. On the final autoradiogram, each band represents a nucleotide position where a break was induced, and the intensity of the band reflects the number of DNA molecules with ligatable ends terminating at that position.
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Coding of Hemolysins Within the Ribosomal RNA Repeat on a Plasmid in *Entamoeba histolytica*

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The pathogenesis of amoebic dysentery is a result of cytolysis of the colonic mucosa by the parasitic protozoan *Entamoeba histolytica*. The cytolysis results in extensive local ulceration and allows the amoeba to penetrate and metastasize to distant sites. Factors involved in this process were defined with three clones that express hemolytic activities in *Escherichia coli*. These potential amoebic virulence determinants were also toxic to human colonic epithelial cells, the primary cellular targets in amoebal invasion of the large intestine. The coding sequences for the hemolysins were close to each other on a 2.6-kilobase segment of a 25-kilobase extrachromosomal DNA element. The structural genes for the hemolysins were within inverted repeats that encode ribosomal RNAs.

Amoebic dysentery results from destruction of the colonic epithelium by the human pathogen *Entamoeba histolytica*. The parasite resides primarily in the large intestine, where it colonizes mucosal surfaces (*1*). Penetration of the colon wall may lead to spread of the amoebae to other organs, most commonly causing hepatic abscesses, and dissemination of the disease (*2*). To invade and cause disease, the amoebae must first penetrate the mucus blanket and adhere to the intestinal epithelium (*3*). Second, the pathogen must degrade the extracellular matrix to release anchored cells. Finally, target cells are lysed by a contact-dependent process and the parasites phagocytose cellular debris. Virulent amoebae are also erythrophagocytic and hemolytic (*4*). Amoebic virulence determinants that are involved in lysis of host cells have also been described and these include a pore-forming protein, amoebaporin (*5*), and hemolytic proteins (*6*). Although the gene for amoebaporin has been identified (*7*), the molecular basis for cytolysis by *E. histolytica* has not been defined. To isolate genes that encode lytic proteins and thereby identify factors required for tissue destruction, we assayed for the functional expression of amoebal hemolysins in *Escherichia coli*.

In the isolation of the hemolysin genes, we took advantage of the predicted biological activity of the virulence factor to detect hemolysins produced from a phage expression gene bank in *E. coli*. The gene bank was constructed by partial cleavage of genomic DNA with Eco RI* to produce randomly digested products for ligation into the λ ZAP/pBluescript vector (*8*). Phage lysis of the bacterial host releases the amoebic proteins and makes them available for detection. The hemolysis assays were done directly on bacterial agar plates containing red blood cells (RBCs). With this approach, the genes can be isolated and the virulence factors characterized in *E. coli* without purification of the proteins from the parasite. Small plaques with relatively large clearing zones were selected at two different pH values that correspond to optima of known *E. histolytica* virulence determinants (*9*). We isolated 12 hemolytic clones that were grouped in five classes by restriction enzyme analysis and size of the inserts. The clones isolated at pH 7.5, represented by pHLY5, were all identical, whereas four different classes were found at pH 6.7. A more detailed restriction enzyme analysis of the inserts showed that representative clones from three of the classes overlapped (Fig. 1). The largest insert (pHLY5) was 2.6 kb in size and contained three Eco RI fragments. Two other clones, pHLY1 and

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pHLY4, contained single Eco RI fragments that represent the right- and leftmost end of pHLY5, respectively (Fig. 1). These three clones were chosen for further studies.

We measured the activities of the three original inserts of pHLY1, pHLY4, and pHLY5 by mixing human RBCs with crude bacterial extracts (Fig. 2). The hemolytic activity was found only in extracts of the hemolysin clones and not in extracts of the vector control. The activities varied between batches of extracts, but the inserts always conferred hemolysis that was not less than 2.5 times that of the controls. The observation that both pHLY1 and pHLY4 encode hemolytic activities indicates that more than one hemolysin coding sequence is located on the pHLY5 insert. To further analyze the clones and to localize the structural genes, we deleted DNA segments from both ends of the inserts and tested the remaining DNA for the ability to express hemolytic activity. In the case of pHLY1, DNA to the left of the Afl II site was removed without loss of activity. Similarly, DNA to the right of the Nde I site was deleted with no effect on hemolysis. Finally, the subcloned Afl II–Nde I fragment contained enough information to express hemolytic activity and was defined as the minimal fragment (pHLY1m; Figs. 1 and 2). This minimal fragment was confirmed by deletion analysis of pHLY5 in which the Bgl II–Nde I segment was sufficient to confer activity (Fig. 1). With the same criteria, a minimal fragment was identified on pHLY4 where the activity is encoded between the Afl II and Xho I sites (pHLY4m; Figs. 1 and 2). Additional deletion analysis of pHLY4 showed that residual activity could be expressed from sequences to the left of the Acc I site on this plasmid, indicating that the structural gene spans this site (*10*). Removal of DNA from pHLY5 that corresponds to both pHLY1 and pHLY4 left a central fragment that was also positive for hemo-

Table 1. Expression of hemolysins in *E. histolytica*. Expression was determined by ELISA with extracts prepared from bacteria carrying different plasmids and an antiserum against whole amoebae (strain HM1). The average relative reactivity and the range of reactivity (interval) of the extracts from four experiments are shown with the vector extract as a reference. The ELISA was done with standard methodology with goat antibody to rabbit (anti-rabbit) conjugated to alkaline phosphate as the detector system.

Extract	Relative reactivity	Interval
pHLY1	1.2	1.0–1.4
pHLY1m	1.5	1.2–1.9
pHLY4	1.3	1.0–1.5
pHLY4m	1.6	1.4–1.8
pHLY5	2.3	2.2–2.4
pHLY5mc	1.2	1.0–1.4
Vector (pBS)	1.0	

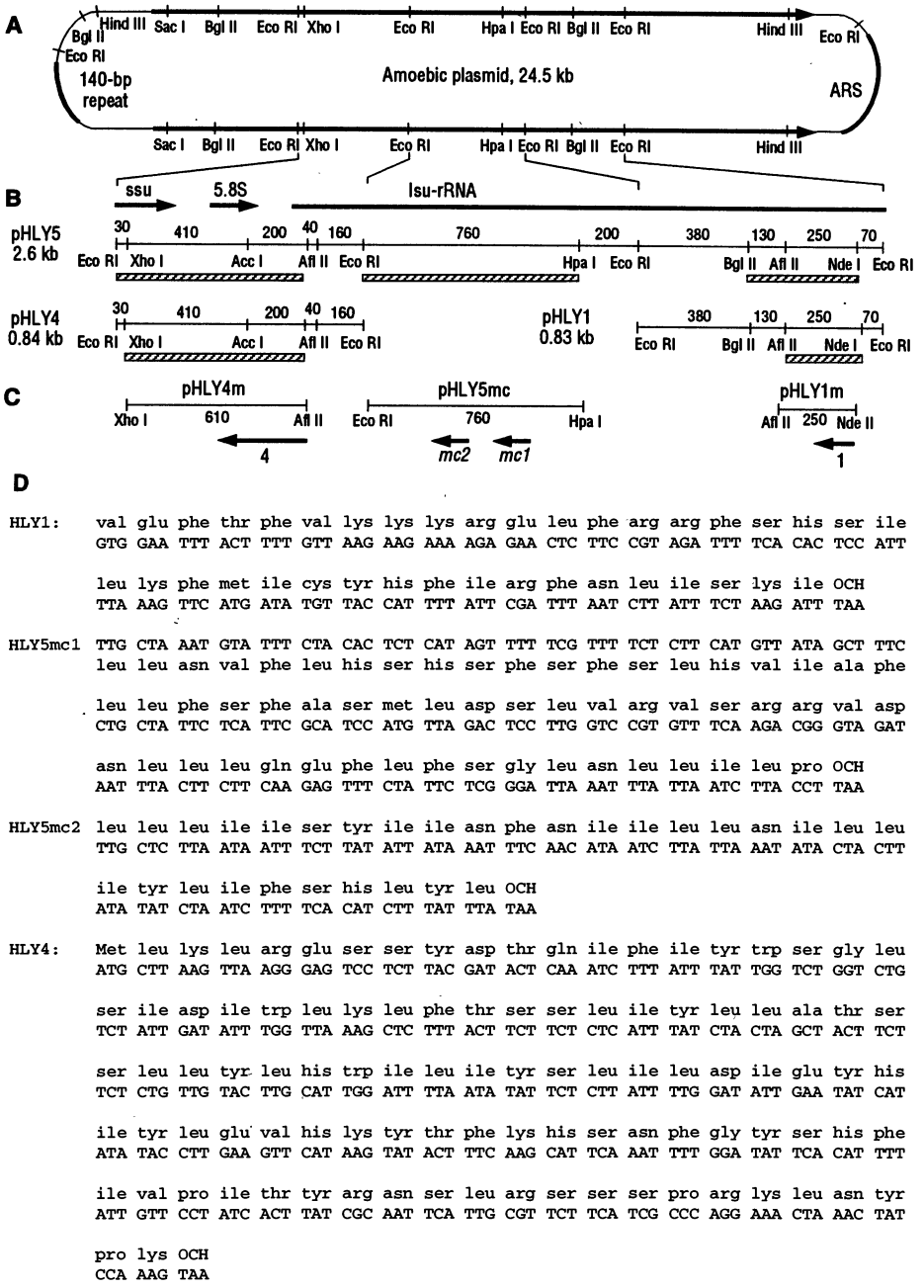

Fig. 1. Physical and genetic map of the rDNA plasmid and the virulence locus. (**A**) The physical map of the circular plasmid is schematically presented with the inverted rRNA repeats depicted as arrows which indicate the direction of transcription [redrawn from Huber *et al.* (*17*)]. The 140-bp repeat is indicated to the left. The physical map is not proportional outside the inverted repeats. (**B**) An enlargement of the central part of the rRNA repeat. The arrows indicate the location of the small subunit (ssu), 5.8*S*, and large subunit (lsu) rRNA genes. The inserts of the hemolytic clones are represented by horizontal lines with plasmid designations and size of the inserts to the left. Restriction enzyme sites and distances between sites are shown below and above the lines, respectively. The hatched bars below the inserts indicate the minimal fragments that express hemolytic activity in different deletion derivatives. (**C**) The minimal fragments of pHLY1m, pHLY4m, and pHLY5mc. Candidate hemolysin genes are indicated with arrows and gene designations are below the arrows. The hemolysin gene positions in the sequence are nucleotides 2498 to 2382 (HLY1), 1428 to 1312 (HLY5mc1), 1197 to 1105 (HLY5mc2), and 644 to 336 (HLY4). Methods are described in (*24, 25, 27*). (**D**) Sequences of the predicted hemolysin genes with deduced amino acids (European Molecular Biology Laboratory Data Library accession number Z29969).

lysin production (pHLY5mc; Figs. 1 and 2). This central fragment, which is distinct from the minimal fragments of pHLY1 and pHLY4, is defined by the Hpa I site and one of the Eco RI sites on pHLY5 (Fig. 1).

We believe that the minimal fragments represent distinct structural genes, as they are well separated in the deletion analysis and splicing is rare in this organism (*11*). This conclusion is supported by the sequence data (Fig. 1D) in which distinct open reading frames (ORFs) were found within each minimal fragment. The ORFs were not fused to any vector gene, which explains the finding that hemolysin expression in *E. coli* is not influenced by isopropylthiogalactoside (IPTG) (*10*). Expression in a heterologous system would normally be dependent on endogenous transcriptional and translational initiation signals, which are provided by the lac cassette of the vector. However, the level of hemolytic activity seems to be independent of vector sequences. To establish whether the hemolysins are expressed in *E. histolytica*, we used an antiserum raised against whole amoebae in an enzyme-linked immunosorbent assay (ELISA) where extracts from the clones were used as antigen. Extract from pHLY5 showed the highest reactivity with the antiserum, whereas extracts from pHLY1m and pHLY4m reacted at an intermediate level (Table 1). The remaining clones did not show reactivity above the background, but it should be noted that the background signal from the crude extract of the vector control was very high which may have obscured weak signals. The results confirm that the hemolysin clones express amoebic antigens in *E. coli*, but it is not conclusive whether all minimal fragments are actively expressed in *E. histolytica*. In conclusion, a total of three minimal fragments expressing hemolytic activities were found within the 2.6-kb insert of pHLY5.

The inserts of the clones were sequenced to identify candidate structural genes for the hemolysins (Fig. 1D). The pHLY4 sequence reveals an ORF of 102 codons that encodes a protein of 12 kD. This ORF lies within the minimal fragment defined in the deletion analysis. It spans the Acc I site and the codon bias is consistent with other *E. histolytica* genes. For the HLY1m minimal fragment, an ORF encoding a protein of 37 amino acids is the most likely structural gene. We have not yet located the structural gene for HLY5mc in which two ORFs of similar size as the HLY1 gene are found in the sequence (Fig. 1). Neither of the candidate hemolysins showed any obvious sequence similarity to known proteins.

We also assessed the possibility that the activities encoded by the hemolysin clones correspond to known virulence factors from *E. histolytica* such as proteases (*12*) and phospholipases (*13*). The results of these experiments show no increased protease or phospholipase activities in the crude extracts. It is therefore unlikely that these enzymes are responsible for the hemolysis. A possible candidate is amoebaporin, a pore-forming protein that causes lysis of target cells (5). However, amoebaporin is not hemolytic for either human or rabbit erythrocytes (5) nor have we found any sequence similarity to amoebaporin in the pHLY5 sequence. Another possibility is the hemolysins described for this organism of which two have been purified (6). However, neither the reported molecular weights nor the amino acid compositions match the candidate hemolysins from pHLY5. Instead, we propose that the gene products are virulence factors not previously described for *E. histolytica*.

Our analysis of the sequence of the compactly organized hemolysin locus revealed that it was identical to that of the *E. histolytica* ribosomal RNA (rRNA) repeat (*14*). The HLY4 coding sequence is located in the intergenic region between the genes for the small subunit RNA and the 5.8S rRNA. Transcription of HLY4 is in the opposite direction to the rRNAs, and the ends of the genes overlap each other (Fig. 1). This is similar to the gene organization of the rRNA repeat from another parasitic protozoan, *Giardia lamblia*, in which an antigen-coding gene is found at the same position (*15*). The HLY1m and HLY5mc minimal fragments both overlap the large subunit rRNA gene, and the candidate gene for HLY1m is transcribed in the same direction as

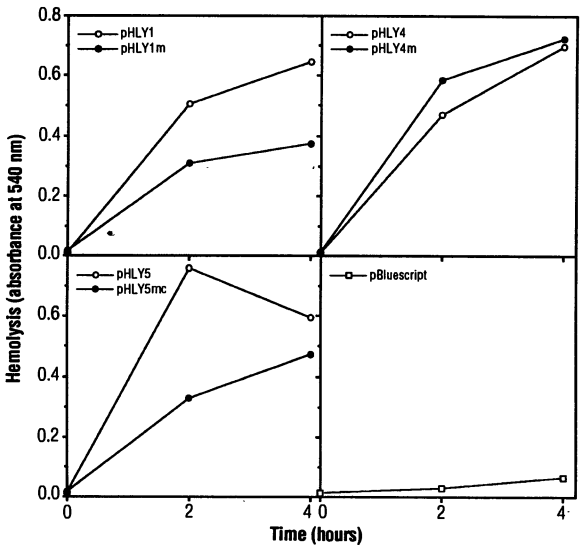

Fig. 2. Hemolysis with recombinant extracts. The amount of lysis of RBCs by crude extracts was determined by absorbtion at 540 nm (*y* axis). Time points were taken at 0, 2, and 4 hours (*x* axis). The vector pBluescript was used as a negative control. Plasmid constructs from which the hemolytic activities are expressed are shown in Fig. 1; m refers to the minimal fragment of the respective original plasmid and mc represents the centrally located minimal fragment of pHLY5. Methods are described in (*26*).

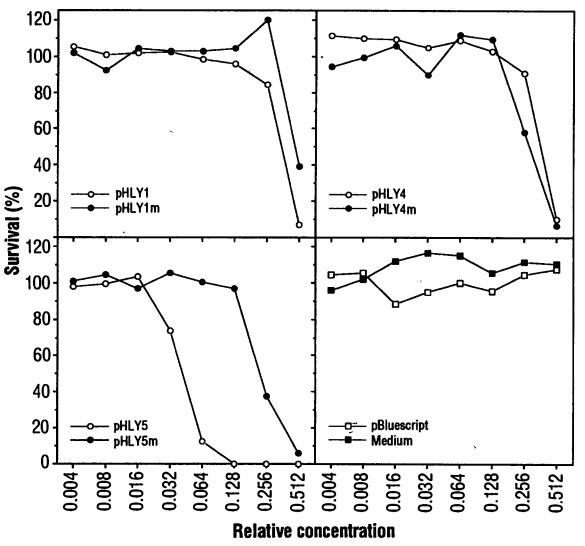

Fig. 3. Cytotoxicity of recombinant extracts. The cytotoxic effect of different concentrations of crude extracts was determined with MTT as an indicator for viability of Caco-2 cells (*19*). Cell viability is shown on the *y* axis and is expressed as a percentage, where 100% survival is an average of the values obtained from cells exposed to extracts from the vector control. Values from cells only grown in medium are shown as a comparison and extract from the pBluescript vector was included as a control. The dilution factor of the crude extracts are given on the *x* axis. Plasmid constructs from which the hemolytic activities are expressed are shown in Fig. 1; m refers to minimal fragment of the respective original plasmid. Methods are described in (*27*).

HLY4 (Fig. 1). It has not been possible to assign a specific ORF as a candidate for HLY5mc, but we assume that this gene is transcribed in the same orientation as the other hemolysin genes. This can be compared with the *Chlamydomonas* mitochondrial genome, in which genes encoding rRNA and respiratory functions are cotranscribed from both strands of the rDNA (*16*). The *E. histolytica* rDNA repeat, and hence the virulence locus, is located on an extrachromosomal element. This plasmid is present in ~200 copies per cell and carries most, if not all, of the rRNA genes of this organism (*17*). A feature of the plasmid is a 140-bp repeated element which has been suggested to be involved in expression of the rRNA (*18*). This 140-bp repeat has also been shown to differ between clinical isolates of *E. histolytica*, and this variation in primary sequence correlates with virulence (*18*). This suggests that the 140-bp repeat may be involved in the expression of the cytolysin genes, but this remains to be established.

To determine if the hemolysins could disrupt a host cell type more relevant to the pathology in the large intestine, we chose the intestinal mucosal cell line Caco-2. This cell line originates from a human colonic carcinoma and resembles polarized mucosal epithelial cells. The effect of hemolytic extracts on the Caco-2 cells was tested in an assay that measures cellular integrity by detecting the activity of a mitochondrial enzyme with a chromogenic substrate (MTT) (*19*). In these experiments, cells exposed to extract from the vector control showed no reduction in viability, whereas decreased viability was observed for cells exposed to extracts from the original hemolytic clones and the minimal fragments (Fig. 3). Microscopic observations showed that the Caco-2 cells exposed to high concentrations of active extract had disintegrated, and we attribute this to the lytic activities defined in the hemolysis assays. The extract from pHLY5 is toxic at higher dilutions than are extracts from the other plasmids or minimal fragments, which may reflect the presence of multiple activities. However, the major point is that the hemolysins are toxic to epithelial cells.

An estimated 500 million people are infected with *E. histolytica* in the world today, but only ~10% of these show symptoms of invasive disease. This discrepancy has been ascribed to differences in amoebic lineages. With isoenzyme typing *E. histolytica* have been divided into pathogenic and nonpathogenic zymodemes (strains). This division has been associated with differences in amoebic DNA sequences (*20*), including the 140-bp repeat of the plasmid (*18*). Differences were also seen when small subunit rRNA was used to probe for restriction enzyme polymorphisms in clinical isolates, and structural differences were associated with clinical manifestations (*21*). Polymorphisms within the rRNA repeat include the hemolysin-coding parts of the amoebic plasmid as shown by Cruz-Reyes *et al.* (*22*), who identified a restriction enzyme dimorphism in the same fragment that we have identified as HLY4. Furthermore, the HLY4 hemolysin gene is present in the pathogenic HM1 *E. histolytica* strain which has been used in study but not found in two other isolates that are not pathogenic in animal models (*23*). The findings presented here establish a direct link between rDNA and virulence genes that explains how differences in the rRNA repeat sequences can influence the course of an amoebic infection.

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26. In the plate screening assays for hemolysis, RBCs obtained from rat were added to soft agar at a final concentration of 2% together with samples of the λ gene bank and bacterial cells. Plates were incubated at 37°C for 6 to 12 hours with continuous monitoring for the appearance of clearing zones around the plaques. To maintain different pH in different assays, we added either Pipes at pH 6.7 or tris-HCl at pH 7.6 to a final concentration of 50 mM. After isolation of individual plaques, phages were converted into plasmids with the excision method described by the supplier (Stratagene). For the liquid assays, the bacteria were grown overnight, centrifuged, and resuspended in tris-buffered saline (TBS) at a density of 50 optical density (OD) units (600 nm). The bacterial suspension was sonicated (four times, 15 s each), and debris was removed by centrifugation. The resulting extract was mixed with a 2% suspension of human RBCs in TBS supplemented with 2 mM $MgCl_2$ and 0.2 mM $CaCl_2$. The total volume was 1 ml and the amount of extract was 200 μl. The mixtures were incubated at 37°C, and samples were taken at 0, 2, and 4 hours. Intact RBCs were removed by centrifugation, and absorbance at 540 nm was used to quantitate lysis.
27. For the cytolysis tests, extracts were produced in the same way as for hemolysis except that the bacteria were resuspended to 100 OD units (600 nm) in Dulbecco's modified Eagle's medium (DMEM) before sonication. The Caco-2 cells were maintained in DMEM with 10% fetal calf serum in 10% CO_2. In the cytolysis assays, the Caco-2 cell line was grown in microtiter plates for 24 to 48 hours, and confluent cell layers were exposed to serial dilutions of crude extracts from the hemolytic clones. After a 2-hour incubation with extract, the cells were washed twice with Hanks balanced salt solution and then incubated for 30 to 45 min in the same buffer with MTT (1 mg/ml) which is a chromogenic substrate for mitochondrial dehydrogenases and an indicator for cell integrity (*13*). The color precipitate was solubilized with acidic butanol and SDS. The color reaction was measured at 590 nm, and high readings indicate viable intact cells and low readings are manifestations of cell death.

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