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Outbreak of Ceftazidime Resistance Due to a Novel Extended-Spectrum β-Lactamase in Isolates from Cancer Patients

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Ceftazidime is widely used in the therapy of infectious complications in neutropenic patients. We studied an outbreak of ceftazidime-resistant gram-negative bacillary infections in pediatric cancer patients receiving empirical ceftazidime therapy for neutropenic fever. Fourteen isolates (12 *Klebsiella pneumoniae* and 2 *Escherichia coli*) from 13 patients were studied. Specimens were obtained from multiple clinical sites including blood, urine, throat, and lung. The organisms were resistant to ceftazidime, aztreonam, and penicillins but remained susceptible to cephamycins and imipenem. All resistant isolates produced a novel β-lactamase (TEM-26) with a pI of approximately 5.58, which was transferred by transformation to *E. coli* on a 7.9-kb nonconjugative plasmid which cotransferred resistance to trimethoprim-sulfamethoxazole. This enzyme readily hydrolyzed ceftazidime, aztreonam, and penicillins in a spectrophotometric assay. DNA sequencing data suggest that TEM-26 is derived from TEM-1.

Profound neutropenia is a frequent complication of chemotherapy for malignant diseases, resulting in substantial risk for bacterial infections. Some of the success of modern cancer chemotherapy is due to early empirical treatment of febrile, neutropenic patients with broad-spectrum antibiotics directed primarily against gram-negative organisms, including *Escherichia coli* and *Klebsiella*, *Enterobacter*, and *Pseudomonas* species.

Ceftazidime is an extended-spectrum cephalosporin antibiotic with activity against most gram-negative organisms, including *Pseudomonas aeruginosa*, and many gram-positive organisms. In some studies ceftazidime has been shown to be effective as empirical monotherapy in neutropenic patients with cancer and unexplained fever (15, 16). A few gram-negative organisms resistant to ceftazidime, particularly *Pseudomonas* isolates, were recovered sporadically in some of these clinical studies, but most organisms remained susceptible. Outbreaks and sporadic cases of infections by ceftazidime-resistant organisms containing plasmid-mediated β-lactamases have been described in Europe, the United States, and elsewhere (5, 13, 14, 19, 20).

Ceftazidime monotherapy was introduced for empirical therapy of fever and neutropenia in children with cancer at the Children's Hospital at Stanford in 1988. Gram-negative organisms causing infection in this population had been uniformly susceptible to ceftazidime until the recent occurrence of the outbreak described in this report.

The purposes of this study were to investigate the epidemiology of infections caused by these ceftazidime-resistant organisms and to determine the molecular mechanism of the resistance.

MATERIALS AND METHODS

Patient population. Infections with ceftazidime-resistant organisms were observed among pediatric oncology patients who were admitted to the Children's Hospital at Stanford for management of fever and neutropenia beginning in June 1989. These patients were generally evaluated initially in the Pediatric Oncology Clinic, and most of them were cared for on a 13-bed inpatient unit reserved for the treatment of children with cancer. Some children were cared for on a similar unit with a mixture of general pediatric patients.

Fever was defined as two oral temperature above 38°C 4 h apart or one temperature above 38.5°C. Neutropenia was defined as absolute neutrophil count of less than 500 (polymorphonuclear leukocytes and band forms per cubic millimeter). The initial evaluation of these patients included a complete blood count, chest radiogram, and cultures of blood, oropharynx, urine, and stool. Empirical antibiotic therapy consisted of 50 mg of ceftazidime per kg given intravenously every 8 h, with a maximum dose of 2 g every 8 h. Some patients also received 10 mg of vancomycin per kg every 6 h and/or amphotericin as part of the empirical regimen. The usual course of treatment for patients whose cultures yielded an organism susceptible to ceftazidime was 10 to 14 days. In most patients whose cultures remained negative, ceftazidime therapy was continued until fever and neutropenia had resolved, with a usual duration of 5 to 10 days.

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Microbiologic surveillance of pediatric oncology patients for ceftazidime-resistant organisms was carried out in June 1990. Urine, rectal, and throat swabs from pediatric oncology patients admitted with the diagnosis of fever and neutropenia were screened for ceftazidime-resistant organisms on selective media as described below.

Laboratory methods. (i) Clinical. Blood, oropharyngeal, urine, and stool cultures were held for 1 week and examined daily; blind subcultures of blood were done on days 1 and 2 after collection. Organism identification and susceptibility testing were initially performed by using the 60 AMS (Vitek Systems, Hazelwood, Mo.). Agar dilution MIC determinations were later performed on isolates and transformants.

Surveillance for ceftazidime-resistant enteric organisms was carried out by culturing rectal, urine, or throat samples onto MacConkey agar plates containing 10 μg of ceftazidime per ml.

(ii) Laboratory. Transformation studies were conducted by using calcium chloride-competent cells and plasmid DNA that had been prepared by alkali lysis (9). Transformants were selected on SOB medium (10)–20 mM $MgSO_4$ containing 10 μg of ceftazidime per ml. The plasmid DNA of all isolates was detected by the rapid alkali lysis technique of Portnoy et al. (17). Samples were subjected to electrophoresis through 0.5% agarose at 12 V/cm. Plasmid size was determined by comparison with molecular weight standards.

A DNA probe for TEM genes was kindly provided by R. Levesque. This probe is a 242-bp *Bgl*I-*Hin*cII fragment which is internal to the β-lactamase gene of pBR322 (8). DNA was labeled with ^{32}P in vitro by using a random-primer method (Pharmacia LKB Biotechnology Inc.) and hybridized under stringent conditions to DNA immobilized onto nitrocellulose.

Isoelectric focusing of β-lactamases was conducted by the method of Matthew et al. (11), using a Hoefer Isobox with prepared Pharmacia Ampholine PAG plates (pH 4.0 to 6.5). Gels were stained with the chromogenic cephalosporin nitrocefin.

A β-lactamase of pI 5.58 was purified from the *E. coli* transformant harboring plasmid pJPQ101 (bla_{TEM-26}) by using Sephadex G75 and boronic acid affinity chromatography (19). Reference β-lactamases were purified from the following strains by using the same procedure: *E. coli* 2639E for the TEM-9 enzyme (2), *Klebsiella pneumoniae* KC2 (plasmid pJPQ 100) for the TEM-10 β-lactamase (19), and *E. coli* JM109 (plasmid pRDD006) for the E104K/R164S enzyme (24). These enzymes were purified to greater than 90% homogeneity as determined by isoelectric focusing with Coomassie blue as a protein stain and SQ 24,902, a chromogenic cephalosporin, to assay for β-lactamase activity. Purified enzymes were used for the hydrolysis and inhibition studies.

Initial hydrolysis rates were monitored spectrophotometrically at 25°C in 0.1 M phosphate buffer (pH 7.0). The computer program ENZPACK (Biosoft) was used to calculate kinetic parameters. Usually triplicate determinations were made for each substrate. Substrates were assayed as a group on at least 2 days. The mean coefficients of variation were 39% for relative V_{max} values and 32% for K_m values.

DNA sequencing. The DNA sequence of the bla_{TEM} gene encoding the TEM β-lactamase variant enzyme was determined by using the dideoxy-chain termination method (21) applied to a single-stranded DNA template. The single-stranded DNA template was obtained by subcloning the bacteriophage f1 origin of DNA replication on a DNA restriction endonuclease fragment on a 466-bp *Bam*HI restriction endonuclease site in the 7.9-kg nonconjugative plasmid containing the bla_{TEM} gene. The resulting plasmid was transformed by a calcium chloride procedure (10) into *E. coli* TG1 [*Δ(lac-pro) supE thi hsdDS/F' traD36 proAB lacL lacZM15*]. Single-stranded DNA was generated from transformants as described by Maniatis et al. (10). Custom-synthesized oligonucleotides were used to prime synthesis from defined sites within the bla_{TEM} gene. The nucleotide sequence of one strand of the coding region of the bla_{TEM} gene was determined.

RESULTS

Epidemiology of ceftazidime-resistant enteric organisms among pediatric oncology patients. During the period from January 1988 to March 1990, 14 clinical isolates of ceftazidime-resistant *K. pneumoniae* (12 isolates) or *E. coli* (2 isolates) were obtained from 13 pediatric oncology patients with fever and neutropenia.

The initial ceftazidime-resistant *K. pneumoniae* isolate was cultured from the urine of a patient who was not responding to ceftazidime monotherapy. Over the next 26 months *K. pneumoniae* isolates resistant to ceftazidime were obtained from blood, urine, and upper and lower respiratory tract specimens from 10 other patients. During the same period, three ceftazidime-susceptible *Klebsiella* isolates were obtained from three oncology patients. However, two of these three patients later acquired infections due to ceftazidime-resistant *Klebsiella* isolates. *K. pneumoniae* was recovered from urine specimens from two additional patients, but susceptibility testing was not performed.

Also during this time interval, *K. pneumoniae* was isolated from cultures of specimens from various sites from nine children who were hospitalized for nononcologic diseases. Seven of these isolates were tested, and all were susceptible to ceftazidime. All patients with ceftazidime-resistant *K. pneumoniae* infections were oncology patients who had been treated with ceftazidime for episodes of fever and neutropenia from 1 to 12 times previously with a total ceftazidime dosage ranging from 1 to 19.25 g/kg (21 to 382 doses).

Between January 1988 and March 1990, two *E. coli* isolates resistant to ceftazidime were recovered from pediatric oncology patients. During the same interval, 57 other *E. coli* strains from throughout the hospital were found to be susceptible to ceftazidime.

The occurrence of the ceftazidime-resistant organisms was not totally sporadic; there were three distinct clusters. Nevertheless, retrospective chart review did not reveal a common source for the outbreak. There was evidence for colonization of patients by ceftazidime-resistant organisms, since one patient had ceftazidime-resistant *K. pneumoniae* in the stool while receiving tobramycin and another patient experienced colonization of the respiratory tract with ceftazidime-resistant *K. pneumoniae* which was not eradicated by a course of amikacin despite in vitro susceptibility. Six patients acquired or developed ceftazidime-resistant *K. pneumoniae* infections while receiving ceftazidime. Another patient initially had a susceptible *K. pneumoniae* isolate in a blood culture on one admission for neutropenic fever but had a ceftazidime-resistant *K. pneumoniae* isolate in a blood culture on a subsequent admission almost 2 months later.

The antibiograms of the ceftazidime-resistant *K. pneumoniae* and *E. coli* isolates were compared with those of the susceptible isolates. All ceftazidime-resistant isolates were cross-resistant to aztreonam, ureidopenicillins, and ticarcillin-clavulanate. They were susceptible to cephamycins and

TABLE 1. Hydrolysis of beta-lactam antibiotics by the TEM-26 β-lactamase

Antibiotic	TEM-23		TEM-9[a]		TEM-10[a]		E104K/R164S[b]	
	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)	Relative V_{max}	K_m (μM)
Benzylpenicillin	100	6.5	100	35	100	4.8	100	5.8
Carbenicillin	32	0.91	19	15	36	5.4	ND[c]	ND
Cloxacillin	18	30	8.7	18	17	46	ND	ND
Cephaloridine	120	103	67	120	77	68	94	90
Cefotaxime	7.5	49	12	450	1.6	43	6.3	84
Ceftazidime	290	290	35	78	68	170	180	150
Aztreonam	49	89	40	490	10	38	31	64

[a] Data from reference 2.
[b] Data from reference 24.
[c] ND, not determined.

imipenem. Of 14 ceftazidime-resistant organisms, 9 were cross-resistant to cefazolin, while all 14 isolates were resistant to trimethoprim-sulfamethoxazole. All of the oncology patients had received trimethoprim-sulfamethoxazole for pneumocystis prophylaxis. One isolate was resistant to gentamicin and tobramycin.

In contrast, the ceftazidime-susceptible *K. pneumoniae* isolates were uniformly susceptible to cefazolin, and 10 of 14 were inhibited by trimethoprim-sulfamethoxazole. The ceftazidime-susceptible isolates which were resistant to trimethoprim-sulfamethoxazole were from patients who had been treated with the latter drug extensively in the past.

Some of these patients with resistant organisms were rescreened several months later. Of five patients whose urine specimens previously had grown ceftazidime-resistant organisms, three had persistent asymptomatic bacteriuria with ceftazidime-resistant enteric organisms and two had sterile cultures. Surveillance of other pediatric oncology patients for colonization with ceftazidime-resistant enteric organisms showed such organisms from rectal swabs or urine in 3 of 83 cultures obtained from 35 patients who were admitted for fever and neutropenia.

Clinical outcome of infections caused by ceftazidime-resistant enteric organisms. Of our 13 patients, 4 had bacteremia. Ceftazidime-resistant organisms caused or contributed to the deaths of two of these patients, both of whom developed septic shock. One of these patients had relapsed acute myelogenous leukemia and profound neutropenia, occupied the bed next to our index case, and developed *Klebsiella* bacteremia 10 days after the roommate. The other patient who died as a consequence of infection with ceftazidime-resistant *K. pneumoniae* had acute lymphoblastic leukemia in partial remission. He acquired resistant organisms during a bone marrow transplant, became bacteremic, developed septic shock, and died. The antibiotic coverage of the other two patients with bacteremia was modified by the addition of tobramycin either empirically or after culture results were obtained. Neither patient developed septic shock, and both survived.

Seven patients had ceftazidime-resistant organisms limited to their urine cultures. Of these, only one had urinary symptoms and a colony count of greater than 100,000/ml. The other patients had ceftazidime-resistant *K. pneumoniae* or *E. coli* isolates with colony counts of less than 100,000 colonies per ml but with no symptoms except fever. Two patients required treatment with supplemental antibiotics besides ceftazidime to eradicate the resistant strains. However, the other five patients with resistant organisms in the urine were treated with ceftazidime alone and had subsequent negative cultures or greatly reduced colony counts.

Ceftazidime-resistant *K. pneumoniae* was found in swabs from the oropharynx of two patients, one of whom also had a positive bronchoscopic lavage culture. Each of these patients had signs and symptoms of pneumonia, which resolved while the patient was receiving ceftazidime despite in vitro resistance of the organism.

Although the source of the epidemic was not identified, the practice of treating febrile neutropenic children with empirical ceftazidime monotherapy was discontinued in September 1990, at which time empirical therapy was changed to amikacin, azlocillin, and nafcillin. No further cases have been detected as of June 1992.

Investigation of the molecular basis for ceftazidime resistance. Agarose gel electrophoresis revealed that the resistant isolates all carried a 7.9-kb plasmid (pJPQ101). In addition, the only isolate which was resistant to gentamicin and tobramycin had an additional 18.4-kb plasmid. Multiple attempts were made to transfer the 7.9-kb plasmid by conjugation, but these were unsuccessful. It was readily transferred by transformation, however. DNA hybridization studies revealed that this plasmid contained a TEM β-lactamase gene. Transformants also acquired resistance to trimethoprim-sulfamethoxazole.

All resistant organisms expressed a β-lactamase with a pI of 5.58. This pI proved indistinguishable from that of several other TEM-class extended-spectrum enzymes. However, on the basis of the biochemical and genetic evidence presented below, we believe that this enzyme is unique.

Properties of the enzyme. As shown in Table 1, the purified enzyme hydrolyzed ceftazidime quite readily. Aztreonam, carbenicillin, and cloxacillin were also rapidly hydrolyzed by this enzyme. Cefotaxime was only slightly more stable, with hydrolysis rates approximately 7.5% that of benzylpenicillin. This pattern of hydrolysis rates was similar to that of the extended-spectrum β-lactamases TEM-9 and TEM-10, which hydrolyze aztreonam and ceftazidime faster than they hydrolyze cefotaxime. However, the properties of this enzyme more closely resembled those of the genetically constructed TEM-1 derivative, E104K/R164S (2, 24). The enzyme was inhibited by clavulanic acid, sulbactam, and tazobactam, with 50% inhibitory concentrations (IC_{50}) of 2.0, 85, and 34 ng/ml, respectively.

The inhibition profile for the novel enzyme was similar to that of the other extended-spectrum enzymes. IC_{50}s of clavulanic acid were considerably lower than that observed for the TEM-1 enzyme (Table 2). Tazobactam inhibition was

TABLE 2. Inhibition of β-lactamases by clavulanic acid, tazobactam, and sulbactam

Enzyme	IC_{50} (nM) of:		
	Clavulanic acid	Tazobactam	Sulbactam
TEM-1	90	97	900
TEM-9	9.0[a]	77	270[a]
TEM-10	4.4[b]	87	940[b]
TEM-26	8.4	77	350
E104K/R164S	6.2	63	320

[a] Data from reference 2.
[b] Data from reference 18.

TABLE 3. Nucleotide substitutions in plasmids producing TEM-1, TEM-26, TEM-9, and E104K/R164S β-lactamases

Nucleotide no.	Nucleotide in[a]			
	TEM-1	TEM-9	TEM-26	E104K/R164S[b]
226	T	T	C	T
263	C (Leu-19)	C (Phe-19)	T (Leu-19)	C (Leu-19)
346	G	G	A	G
512	G (Glu-102)	A (Lys-102)	A (Lys-102)	A (Lys-102)
604	T	T	G	T
682	T	T	C	T
692	C (Arg-162)	A (Ser-162)	A (Ser-162)	A (Ser-162)
925	G	G	A	G
990	C (Thr-261)	C (Met-261)	T (Thr-261)	C (Thr-261)

[a] Amino acid substitutions are shown in parentheses.
[b] The genetically constructed strain was numbered according to Ambler (1). Amino acids 102 and 162 in the Sutcliffe system are equivalent to 104 and 164 in the Ambler system.

comparable for all the enzymes. The IC_{50}s of sulbactam for the extended-spectrum enzymes, with the exception of TEM-10, were lower than those for the TEM-1 enzyme. Sulbactam was the weakest inhibitor tested.

DNA sequencing. To obtain the amino acid sequence of the β-lactamase responsible for the ceftazidime resistance, we determined the coding sequence of the gene (bla_{TEM}) encoding the β-lactamase on the 7.9-kb plasmid. The results confirmed the DNA hybridization results in that the amino acid sequence is related to the TEM β-lactamase. Although it is a member of the TEM β-lactamase family of enzymes, the enzyme encoded by the 7.9-kb plasmid (here called TEM-26) has a novel amino acid sequence. As seen in Table 2, the amino acid sequence of the TEM-26 enzyme closely resembles that of the TEM-9 extended-spectrum β-lactamase, which is also capable of hydrolyzing ceftazidime and was first identified in ceftazidime-resistant clinical isolates in England (9, 25). The only differences in amino acid sequence between these enzymes are the Phe-19 and Met-261 substitutions found in TEM-9 but not TEM-26. The Phe-19 substitution is in the signal sequence and is thus not a part of the mature enzyme (14). In contrast, the Lys-102 and Ser-162 substitutions are found in many extended-spectrum β-lactamases and have been shown to be directly responsible for the ability of TEM β-lactamase to cleave ceftazidime (24). In fact, the mutations observed are identical to those in enzyme E104K/R164S (24), which was constructed from a TEM-1 β-lactamase, an enzyme that originally carried two conservative amino acid modifications (V821 and A182V) compared with pBR322. On the basis of the very high hydrolysis rates for ceftazidime compared with those of the TEM-9 enzyme, one may conclude that residue Met-261 is important in determining the hydrolytic properties of these enzymes.

Although TEM-26 and TEM-9 share the Lys-102 and Ser-162 amino acid substitutions, the DNA sequences of the respective genes contain several differences in the coding regions. Therefore, it is unlikely that the *blaT9* gene is the parent of the TEM-26 gene. This gene is most closely related in nucleotide sequence to the TEM-1-encoding gene $bla_{TEM\text{-}1b}$ derived from the Tn*2* transposon (Table 3) (3). From the sequences, it appears that the TEM-26 gene evolved from the $bla_{TEM\text{-}1b}$ gene. It is important to note that the TEM-1 enzyme encoded by the $bla_{TEM\text{-}1b}$ gene itself does not have activity toward ceftazidime, but under the selective pressure of ceftazidime therapy, two nucleotide substitutions lead to the ceftazidime-hydrolyzing TEM-26 enzyme.

DISCUSSION

Since the publication of the study of Pizzo et al. in 1986 (16), empirical monotherapy with extended-spectrum beta-lactams for febrile neutropenic episodes in cancer patients has become increasingly popular across the United States. This report raises a concern about the safety of this approach. It should be pointed out that there is a substantial problem with ceftazidime resistance among *Enterobacter* and *Pseudomonas aeruginosa* isolates since most of these strains harbor inducible chromosomal β-lactamases. Approximately 40% of all *Enterobacter* strains from a study of intensive care units in the United States are resistant to extended-spectrum cephalosporins, primarily owing to chromosomal β-lactamase activity (6). Treatment failure may occur in this setting owing to selection of spontaneous mutants which hyperproduce chromosomal enzymes (4). Such organisms are typically broadly resistant to beta-lactams other than imipenem.

The epidemic described in this paper is due to a different phenomenon, namely plasmid-mediated β-lactamases that hydrolyze broad-spectrum cephalosporins. Organisms of this type were first described in Europe in 1983 (7) and have since spread rapidly on the European continent and have appeared worldwide, including the United States (5, 14). They have not yet become the prevalent bacteria in most hospitals, but they have caused several large epidemics in intensive care units (1a).

An exhaustive review of the molecular biology of these enzymes is beyond the scope of this paper. Suffice it to say that most of these enzymes belong to the TEM and SHV classes of enzymes. The latter is a native enzyme of *Klebsiella* spp., whereas the former is widely distributed among gram-negative pathogens. For example, about 40% of recent *E. coli* isolates in the United States are ampicillin resistant as a result of the production of TEM class enzymes (6, 12). Cloning and sequencing of the new extended-spectrum plasmid-mediated β-lactamases has demonstrated that they are point mutants arising from genes in these older, well-established classes (22). Consequently, the genetic pool from which these mutants can arise is extraordinarily large.

Molecular modeling studies have shed considerable light on the structure-activity relationships which account for selective resistance due to these enzymes. In contrast to the broad resistance one typically sees as a result of chromosomal enzymes, organisms harboring novel plasmid-mediated enzymes may be more resistant to compounds such as ceftazidime and aztreonam (14). Specific amino acid mutations, which allow for interaction with the aminothiazole-oxime side chain of these compounds and hence proficient

destruction of the beta-lactam ring, have been mapped in these enzymes (23). Thus, such organisms may be resistant to ceftazidime, aztreonam, and extended-spectrum penicillins while remaining susceptible to other beta-lactams, including cefotaxime and ceftriaxone. Organisms of this type are almost invariably susceptible to cephamycins and carbapenems. A interesting and alarming exception is a novel enzyme recently characterized by Papanicolaou et al., which confers broad resistance reminiscent of that seen with chromosomal enzymes (13). This enzyme, called MIR-1, has been shown to be closely related to a chromosomal β-lactamase originating in *Enterobacter* isolates, which has spread on a plasmid among *Klebsiella* isolates, presumably on a transposon.

Two other microbiologic points deserve special mention. First, since some extended-spectrum enzymes may selectively hydrolyze ceftazidime, it is incumbent on clinical microbiology laboratories to test ceftazidime susceptibility specifically against enteric bacteria. The concept of class susceptibility testing for extended-spectrum cephalosporins must be abandoned in light of the spread of these enzymes across the United States. Second, many of these enzymes are carried on broad-host-range plasmids which transfer multiple resistance genes. For example, the plasmid responsible for this outbreak cotransferred resistance to extended-spectrum beta-lactams and trimethoprim-sulfamethoxazole. Some of the plasmids incriminated in other studies have cotransferred resistance to aminoglycosides as well (18). Most of the plasmids described in prior studies have also been much larger (80 kb and above) (14) than pJPQ101.

In summary, we have described an epidemic of plasmid-mediated ceftazidime-resistant enteric gram-negative bacillary infections among children with cancer receiving empirical ceftazidime monotherapy. The increasing prevalence of ceftazidime resistance among gram-negative rods as a result of both chromosomal and plasmid-mediated resistance determinants calls into question the safety of empirical monotherapy with this agent.

ADDENDUM IN PROOF

After submission of this paper, another variant of TEM-1 was described with the amino acid substitutions of Ser for Arg at 162 and Lys for Arg at 237. This corresponds to laboratory mutant R164S/E240K described in reference 24. TEM-23 and TEM-26 would be expected to have similar biochemical and phenotypical properties (G. Vedel, C. Mabilat, B. Goussard, B. Picard, G. Fournier, L. Gilly, G. Paul, and A. Phillipon, FEMS Microbiol. Lett. **93**:161–166, 1992).

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