Appl Microbiol Biotechnol (1984) 20:344–350

Applied and Microbiology Biotechnology

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Mutants of *Myxococcus xanthus* impaired in protein secretion: an approach to study of a secretory mechanism

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Summary. Bacteria able to secrete proteins efficiently into the growth medium occur relatively rarely amongst Gram-negative species. However, the increasing technological interest in protein secretion has focused attention on this process. We have demonstrated that *Myxococcus xanthus* actively secretes protein. The number of proteins secreted is quite large, but the total amount is strictly regulated and remains constant under conditions that change the specific activities of some of the secreted enzymes. Tn5-insertion mutants were obtained which were impaired in what seems to be the control system for protein secretion. Two of the mutants displayed increased levels of extracellular protein.

Introduction

Myxococcus xanthus is a Gram-negative bacterium that has been studied mainly as a procaryotic model for multicellular morphogenesis (Dworkin 1972, Kaiser et al. 1979, Kuner and Kaiser 1982). Another unusual property of this organism has been accorded less consideration: proteins secreted by *M. xanthus* are not accumulated in its periplasm, as happens in other Gram-negative bacteria such as *Escherichia coli*, but are secreted into the growth medium (Kaiser et al. 1979, Haska and Stahl 1971). Such a property is well known among Gram-positive bacteria (Glenn 1976). The current biotechnological interest in protein secretion as an economical mean of production of some engineered proteins has, however, focused attention on how little is known about extracellular protein secretion by Gram-negative bacteria.

Escherichia coli secretes only specialized proteins, such as colicins or haemolysins (Van Tiel-Menkveld et al. 1979, Springer and Goebel 1980). There is, however, still some doubt as to whether this is genuine secretion or, as has been demonstrated for colicins, associated with leakage of proteins from non-viable bacteria. In contrast, some Gram-negative bacteria do clearly secrete proteins, for example, *Aeromonas hydrophila* (Howard and Buckley 1983); *Vibrio alginolyticus* (Bowden et al. 1982) and *Pseudomonas aeruginosa* (Jensen et al. 1980, Wretlind et al. 1977). At present very little is known about the mechanism or regulation of this process (Nielsen et al. 1983).

Previous studies have indicated that *Myxococcus xanthus* does clearly secrete proteins into the medium. Several bacteriolytic (Sudo and Dworkin 1972) and proteolytic enzymes (Gnospelius 1978) have been identified in the growth medium. Since this species is now amenable to genetic analysis (Campos et al. 1978, Kuner and Kaiser 1981), it is therefore possible and of interest to study the mechanism of release of extracellular protein. In addition, this non-pathogenic bacterium can be grown in semi-large scale reactors (Irschik et al. 1983) and it has been used for experiments in genetic engineering (Shimkets et al. 1983, O'Connor and Zusman 1983). This organism might therefore be an alternative to *Bacillus subtilis* as a host for secretion of foreign proteins determined by genes previously cloned into *E. coli*. In this paper we show that the production of extracellular proteins by *Myxococcus xanthus* is true secretion. By using the transposon mutagenesis we were able to obtain mutants impaired in the control of this protein secretion process.

Materials and methods

Organism and phages. The organism used was *Myxococcus xanthus* strains DK 101 (Kuner and Kaiser 1981); CM strains isolated in the laboratory are Tn5 (Kan^r) insertion mutants of DK 101. Phages P1: :Tn5 (Tn5 insertion in phage P1) and P1: :Tn5-132 (phage P1 carrying a Tet^r derivative of transposon Tn5) were kindly provided by Dr. D. Kaiser (Dept. of Biochemistry, Stanford University,

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Stanford, California 94305 USA). The transducing phage MX4ts-hrm isolated by Campos et al. (1978) was a gift of Dr. D Zusman, Dept. Microbiology & Immunology, University of California, Berkeley, California 94720, USA.

Media. The liquid medium CTT has already been described (Dworkin 1962). The CYE solid medium contained per litre: 10 g Casitone (Difco); 5 g Yeast extract; 2 g $MgSO_4$, 7 H_2O; 12 g Agar (Bio Merieux). Top agar was 0.6% agar in Tris-Phosphate-Mg buffer. Antibiotics, when used, were Kanamycin sulphate (Bristol) and Oxytetracyclin chlorhydrate (Roussel-Uclaf).

Transposon mutagenesis and genetical analysis. Stationary cells of strain DK 101 were mixed with phage P1: :Tn5 in CTT medium with 5 mM $CaCl_2$. The mixture was incubated without shaking and plated on CYE supplemented with 25 µg/ml Kanamycin. The plates were overlaid after one night of incubation at 30° C, with soft agar medium containing Kanamycin at a final concentration of 75 µg/ml (no spontaneous mutants were ever found under these conditions).

The presence of Tn5 in the CM strains was checked by the Tn5/Tn5-132 exchange method (Kuner et al. 1981) The Kan^r strains were infected by P1: :Tn5-132 and the Tet^r derivatives were tested for the absence of Kanamycin resistance and for conservation of the original phenotype: they bear a Tn5-132 insertion in the same locus as the original Tn5 insertion. Phage lysates were prepared as described by Campos et al. (1978). Genetic mapping was performed by crossing Tn5-132 exchanged insertion mutants with the phage stocks prepared from the Tn5 insertion mutants. The transduction mixture was spread onto CYE (25 µg/ml Kanamycin) and overlaid with Kanamycin soft agar as described. The emerging transductants were purified to single colonies and checked both for their resistance to Oxytetracyclin and for other markers on test plates.

Plate assays. Cells were transferred to CYE solid medium plates overlaid with 3 ml soft agar containing the reaction substrates: skimmed milk for proteases, heat killed Gram-positive bacteria (*B. Subtilis, lactobacillus sp.*) for bacteriolytic activities, starch for amylase, RNA, or methyl-green DNA (Sigma) for RNase and DNase respectively (Horney and Webster 1974). The activities were revealed with I_2/KI for starch hydrolysis and with perchloroacetic acid for RNA (Lazzaroni and Portalier 1979).

Enzyme assays. The total proteolytic activity was assayed at pH 8 with azocasein as the substrate; 1 U (UA) is defined as the amount of enzyme which hydrolyses 1 mg azocasein per 30 min at 37° C (Millet 1970). Bacteriolytic activity was assayed at pH 7.6 with lyophilized cells of *Micrococcus lysodeicticus* (Sigma) as the substrate; one unit (BEU) was defined as the amount of enzyme which gives a decrease of absorbance: 0.001 (580 nm/min at 37° C (Haska and Stahl 1971). Malate dehydrogenase activity: L-malate: NAD oxydoreductase E.C. 1.1.1.37 was assayed according to the method of Watson and Dworkin (1968). Cell bound acidic phosphatase was assayed on a washed whole-cell suspension according to Torriani (1960) with the following modification: the assay was performed in 0.5 M acetate buffer at pH 5, with para-nitrophenyl phosphate (25 mM) as the substrate; one unit (UP) is defined as the amount of enzyme which catalyses the hydrolysis of 1 nmole of substrate/min at 37° C.

Protein determination. Protein concentration in the broth was measured by the Coomassie blue method (Sedmak and Grossberg 1977).

Analytical isoelectric focusing. Analytical isoelectric focusing in a polyacrylamide gel was performed with an LKB Multiphor system. The ampholine carrier ampholytes used had a pH range of 3.5–9.5

Enzyme solutions were prepared from growth medium of cells spun down at the end of the log phase, then concentrated and dialysed on a PM 10,000 Microprodicon membrane to a protein concentration of 0.5–1 mg/ml. BIO-RAD procedures for precipitating, fixing, staining and destaining the focused proteins were followed as detailed in practical information leaflet entitled "The BIO-RAD Silver Stain" (BIO-RAD England).

Results

Extracellular protein secretion by Myxococcus xanthus

The quantity of protein produced by *Myxococcus xanthus* in the medium was measured during growth of strain DK101 in CTT broth. Figure 1b shows that extracellular protein production per cell was constant throughout the exponential growth. Moreover:

I) there was no increase in the amount of extracellular protein at the end of the exponential growth phase or during the stationary phase; (II) no malatedehydrogenase, a cytoplasmic enzyme, was detected in the extracellular fluid; (III) bacteriolytic activity was found exclusively in the growth medium; (IV) an acidic phosphatase was shown to be 90%

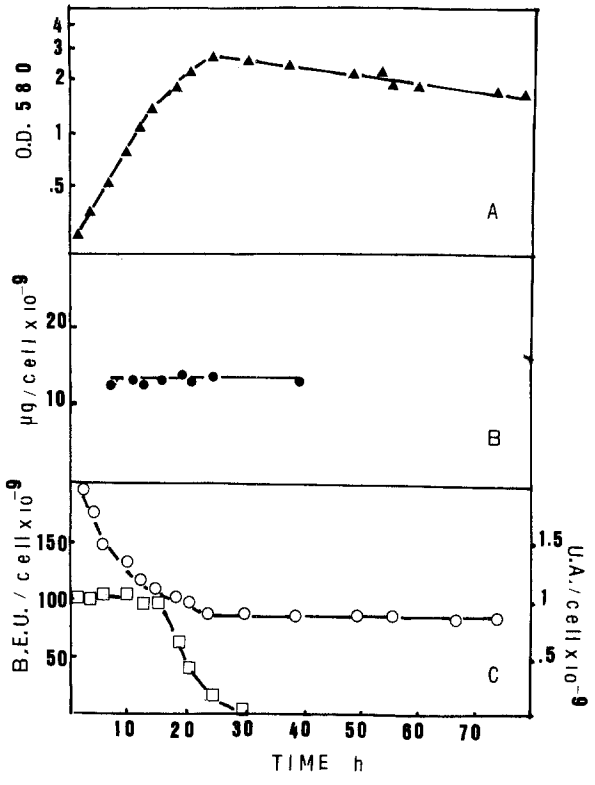

Fig. 1A–C. Extracellular bacteriolytic and proteolytic activities during the growth of strain DK 101 in CTT medium. **A** Growth; **B** protein accumulation in the growth medium per 10^9 bacteria; **C** proteolytic (○———○) and bacteriolytic (□———□) activities per 10^9 bacteria

periplasmic and 10% extracellular, the extracellular proportion remaining constant after the end of exponential growth (Nicaud 1983).

These results show that the extracellular protein did not result from death or damage of for example, the outer membrane of the cells but was probably the result of a true secretory process.

In contrast to the constancy of total extracellular proteins secreted per cell during growth, Fig. 1c shows dramatic modifications in the production per cell of proteolytic and bacteriolytic activities. Changing the casitone concentration of the medium did not affect the total protein production per cell, but resulted in modifications in the proteolytic and bacteriolytic activities excreted per cell (Table 1).

Table 1. Effect of casitone concentration on total extracellular proteins, proteolytic and bacteriolytic activities. These were measured as described in materials and methods. Protein content of the growth medium was assayed during the exponential and early stationary phase. Proteolytic and bacteriolytic activities were assayed at the end of exponential phase and during exponential growth (when the role is constant per cell), respectively

Casitone concentration (g/l)	Extracellular proteins ($\mu g/10^9$ cells)	Proteolytic activity ($UA/10^9$ cells)	Bacteriolytic activity ($BEU/10^9$ cells)
5	12 ± 2	1.30 ± 0.05	35 ± 10
10	13 ± 2	0.85 ± 0.05	100 ± 10
20	13 ± 2	0.50 ± 0.05	65 ± 10

Protein composition of extracellular fluid in wild-type bacteria

The quantity of protein secreted did not exceed 4% of the cell protein with a wild-type strain of *Myxococcus xanthus*. However, large numbers of individual proteins were released into the growth medium. More than 50 bands were detected at the end of exponential growth, when the concentrated growth medium was submitted to electrofocusing (Fig. 2).

In these analyses electrofocusing was preferred to polyacrylamide gel electrophoresis, since the large amount of slime produced in the medium by the bacteria prevented good resolution. Twenty-five major bands can be identified in Fig. 2 and these appear different form the main bands observed when a total cell lysate is submitted to the same electrofocusing (Fig. 2). This result provides further evidence for the existence of an active secretory mechanism.

Isolation and study of secretory mutants of strain DK 101

Derivatives of strain DK 101, harbouring a Tn5 insertion, were tested in the bacteriolytic plate assay in order to compare the size of the halo of lysis surrounding each colony. Colonies displaying a

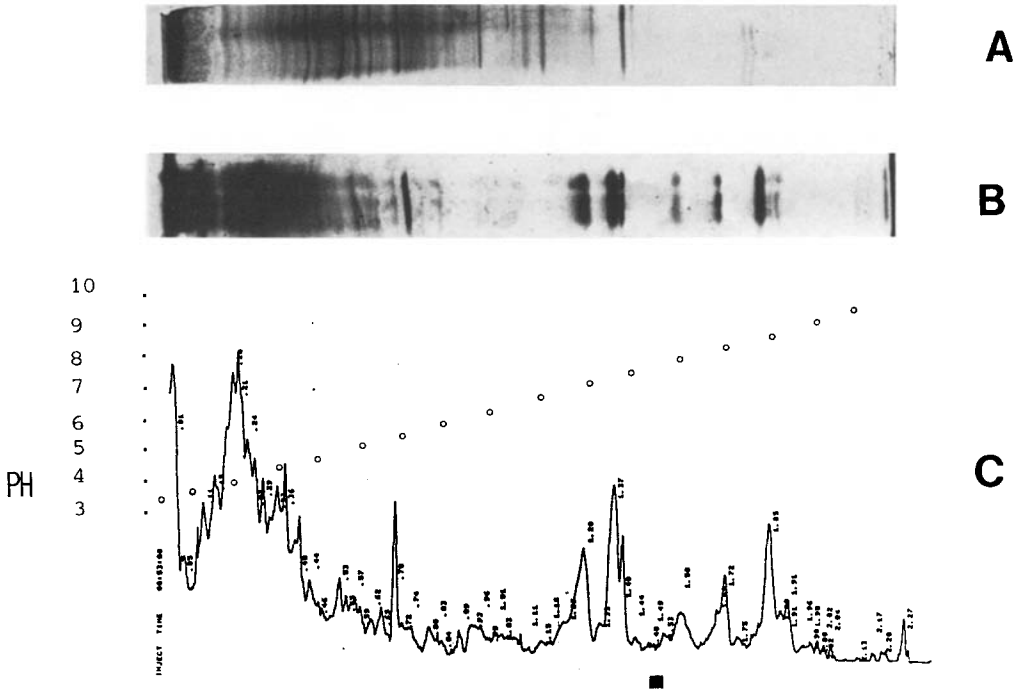

Fig. 2A–C. Electrofocusing pattern of extracellular proteins as compared to intracellular protein of *Myxococcus xanthus*. **A** Intracellular proteins as control; **B** extracellular proteins; **C** densitometric scale of the B profile with a laser Ultorscan LKB 2202

Table 2. Phenotype of Tn5-insertion mutants. Total proteins, proteolytic, bacteriolytic, and acid phosphatase activities were assayed in CTT liquid medium; amylase, DNase, and RNase were assayed on plates. +, wild type; − decreased halo; ++ increased halo (see Materials and methods)

Strains	Total proteins		Proteolytic activity		Bacteriolytic activity		Periplasmic acid phosphatase activity		Amylase activity	DNase activity	RNase activity
	$\mu g/10^9$ cells	%	UA/10^9 cells	%	BEU/10^9 cells	%	UP/10^9 cells	%			
DK101	13	100	0.85	100	100	100	120	100	+	+	+
CM19	8	60	0.24	28	70	70	209	174	−	++	++
CM31	8	60	0.45	53	45	45	154	128	−	++	++
CM33	19	150	1.37	161	150	150	91	76	++	−	−
CM140	19	150	1.23	145	140	140	72	60	++	−	−

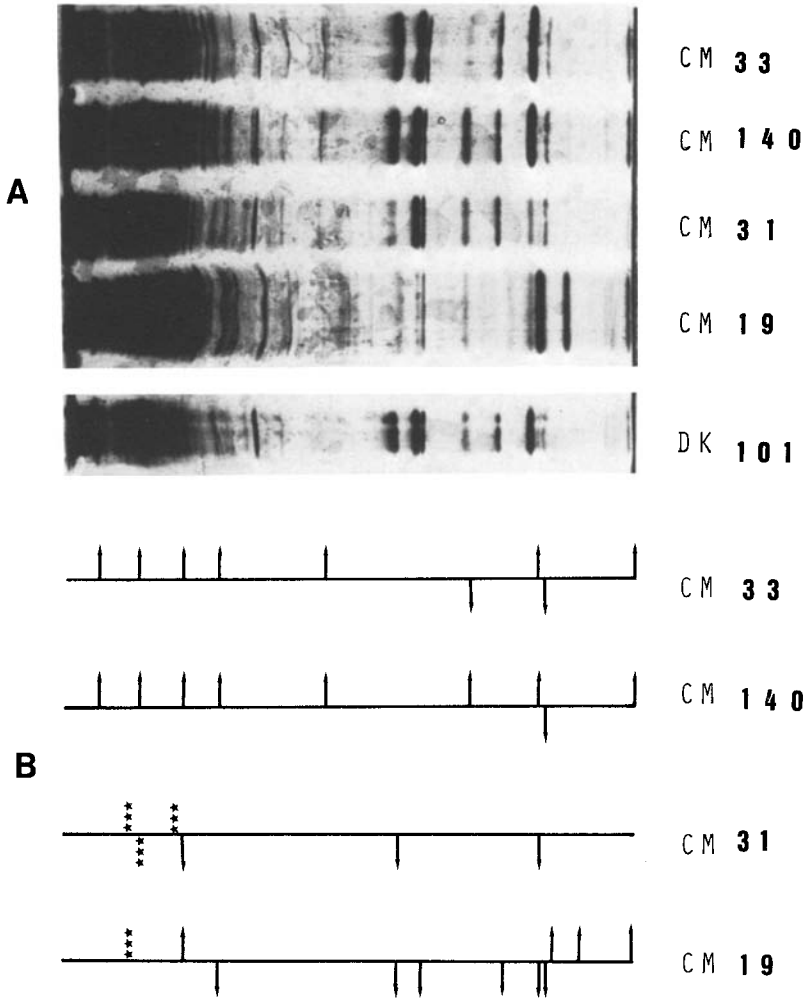

Fig. 3. A Electrofocusing pattern of the extracellular proteins of CM mutants as compared to the wild type strain DK 101. **B** Schematic representation of increase ↑; decrease ↓; appearance*; disappearance⁂ of the major bands as compared to the wild type strain DK 101

modified (decreased or increased) halo compared with that of the parent strain were isolated and further studied. In four strains (out of 1,500 random insertions) the size of the halos appeared also to be affected on the other assayed substrates. The total extracellular production of protein was determined in these strains as well as the level of extracellular proteolytic and bacteriolytic activities.

Table 2 shows that mutants CM 19 and CM 31 had a very low rate of extracellular protein production; the proteolytic, bacteriolytic and amylolytic activities present in the media were also very low. In these mutants there was, however, an increase in RNase and DNase activities, as determined by the halo assay, and also in periplasmic acid phosphatase activity.

By contrast, strains CM 33 and CM 140 produced 50% more extracellular protein, with specific activities (with respect to the total extracellular protein) of protease and bacteriolytic enzymes comparable to those of the wild type.

These observations were confirmed by the analytical electrofocusing of the proteins in the growth media of the four strains (Fig. 3a). The differences between the strains are relatively minor and are summarized in Fig. 3b. Strains CM 33 and CM 140 are nearly identical and show no new protein in the medium compared with the DK 101 strain. CM 19 and CM 31, however, show greater differences from the wild type and also differ from each other, each displaying apparently new bands.

Genetic analysis

Genetic analysis of the mutants was restricted to the two hypoproducing CM 19 and CM 31 strains, since so far we have not succeeded in obtaining transductants or transducing lysates from either CM 33 or CM 140 (a trait that may or may not result from the same mutation).

Tn5/Tn5-132 exchange experiments have shown that both CM 19 and CM 31 possess a Tn5 insertion, since 97%–100% of the Tet^r derivatives have lost the resistance to Kanamycin. This demonstrated that there was a real exchange between the two related transposons. Transduction of strain DK 101 by MX4ts lysates grown on CM 19 or CM 31 yielded Kan^r derivatives which displayed a hyposecretory phenotype similar to the donor parents.

In order to check the genetic linkage between the two insertions, the Tn5-132 derivatives of each strain were transduced with MX4ts lysates grown on the original Tn5 insertion mutants. The Kan^r transductants were all Tet^s in the two homologous crosses, whereas they were Tet^r in the heterologous (CM 19/CM 31) crosses; the Kan^rTet^r/Kan^r ratio (the linkage index) averaged 98% to 100% in each case, showing that CM 19 and CM 31 mutations were not linked. Moreover, the CM 19–CM 31 double insertion transductants thus obtained, displayed proteolysis or bacteriolysis halos (by the plate technique) which were smaller than those of the parent strains. This phenomenon showed that the effects of the two mutations were additive.

Discussion and conclusions

Our results demonstrate that protein production by *Myxococcus xanthus* is the result of actual secretion and does not arise from lysis or damage of the envelopes of some cells. However, although a great number of extracellular proteins are produced during growth in CTT broth, they correspond to a small proportion of total protein production (4% of total cell protein). This result is consistent with previous work showing the inability of dilute suspensions of *Myxococcus xanthus* to grow on casein substrate (Rosenberg et al. 1977). The constancy of the amount of extracellular proteins produced per cell, in contrast to the kinetics of accumulation of the proteolytic and bacteriolytic activities in different media, suggests a mechanism which controls the total amount of protein secreted. A similar situation has been postulated by Coleman for a Gram-positive secreting bacterium (Coleman 1981). The mutants studied here are pleiotropically affected in protein secretion. Although defective as a result of two unlinked insertions, the two hyproproducing mutants have similar properties. Both mutants showed a decrease of 40% in the total amount of secreted protein with no major changes in the electrofocusing pattern of individual bands. Both mutants showed an increase in periplasmic acidic phosphatase activity, but no increase in cell-bound protease or bacteriolytic activities. The minor differences between the extracellular protein patterns of the two strains may be a direct consequence of each mutation or may stem from them indirectly. Thus secretion of different concentrations of specific hydrolases into the medium (casitone) may result in the accumulation of different amounts of particular peptide oligomers which might in turn, if taken up into the cells, affect the regulation at the transcriptional level of other genes coding for secreted proteins. We have observed (Table 1 and Nicaud 1983) that such a regulation of secretory activities does exist.

The two hyperproducing strains are also extremely similar and show the opposite of the activities of the hypoproducers. Thus these mutants secrete 50% more extracellular proteins, contain less periplasmic acidic phosphatase and do not exhibit a decrease of the cell-bound proteolytic activity. The pattern of extracellular proteins in these mutants is very similar to that of the wild type, the few differences involving only the relative quantities of the secreted proteins.

The properties of our mutants indicate that they do not appear to be defective in some non-specific permeation mechanism, as has been described previously for other species. The hypersecretors are different from the "leaky" mutants of *Escherichia coli* described by Lazzaroni and Portalier (1981) since they exhibit neither new extracellular protein bands nor an increase in extracellular acidic phosphatase. The hypoproducers, unlike the *Aeromonas hydrophila* mutants described by Howard and Buckley

(1983), do not accumulate extracellular activities in their periplasm. We suggest that the mutants described here are impaired in the control of their protein secretion.

The acidic phosphatase activity (periplasmic) is clearly increased in the hyposecreting and decreased in the hypersecreting strains. This may reflect some kind of direct regulation of this enzyme by one or more of the secreted proteins or it may indicate that there is an overall control of the total quantity of proteins secreted. Indeed, the regulatory mechanisms involved in a microorganism living upon macromolecular substrates may be very complex. We therefore believe that the elucidiation of the impaired secretory mechanism of these mutants may be more easily achieved by studying the expression of cloned genes under the control of known foreign promoters.

In conclusion, the existence of hypersecreting mutants, as well as the indications that the total quantity of proteins secreted is normally a carefully controlled process may be of importance in view of the potential utilization of *Myxococcus xanthus* for the secretion of foreign proteins. In this context it might be possible to produce high levels of a derepressed protein, and to increase the quantity of the protein secreted by the summation of several hypersecreting mutants.

Acknowledgements. This work was supported by a grant from the "Centre d'Aide au Transfert des Biotechnologies". We thank Dr. Lubochinski for having introduced us to the *Myxobacteriae*, Dr. Kaiser and Dr. Zusman for kindly providing us with phages and strains and Dr. Dworkin for useful discussion.

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Received January 9, 1984