Mode of Action of Colicin S8

By MARIA-ELENA GARCIA,*† ROSA NÁGEL DE ZWAIG AND
JUAN PUIG

*Grupo de Biología Experimental, Facultad de Ciencias, Universidad de los Andes, Apartado
Postal 281 Merida, Venezuela*

(Received 3 August 1981)

The mode of action of colicin S8 has been studied and compared with that of other colicins. Two minutes after the addition of colicin S8 to bacteria a considerable proportion of the colicin is inaccessible to trypsin. Treatment of bacteria with colicin S8 renders them more sensitive to lysis by sodium dodecyl sulphate and also inhibits motility. Like colicins K and E1, colicin S8 provokes lysis of bacteria superinfected with bacteriophage T4. Colicin S8, unlike colicin E2, prevents replication of bacteriophage T4. The incorporation of isoleucine or uracil into bacteria is inhibited by colicin S8 but, unlike colicins K and E1, the effect is multiplicity-dependent. A rapid method of titration of colicin S8 is described. The results are discussed with emphasis on the possible rearrangements at the bacterial surface and the possibility that there is more than one type of specific receptor for colicin S8.

INTRODUCTION

Colicins, bacteriocins produced by some members of the *Enterobacteriaceae*, present a whole spectrum of behaviour involving different levels of the cell surface structure and also biochemical reactions in the cell. As macromolecules they pass across the cell membranes by special mechanisms. Kadner *et al.* (1979) have concluded that at least three fundamental steps are involved: (i) attachment of the colicin molecule to discrete and specific receptors on the cell surface; (ii) conversion of the colicin to a new state or site by an energy-dependent process; and (iii) specific interaction between the colicin and its biochemical target. Much evidence points to the existence of specific receptors on both the cell wall and the cytoplasmic membrane (Smarda, 1975).

With respect to the third step, three major types of biochemical action can be recognized: (i) DNA degradation, e.g. colicin E2 (Buxton & Holland, 1974); (ii) inhibition of protein synthesis, e.g. colicins E3 and D (Bowman *et al.*, 1971); and (iii) inhibition of all macromolecular synthesis and also active transport of amino acids and β -galactosides, e.g. colicins E1, K, Ia, A (Tokuda & Konisky, 1979; Kayalar & Luria, 1979; Nágel de Zwaig, 1969). The effects of colicins E1 and Ia have recently been shown to be derived from their ability to disrupt the energized state of the bacterial cytoplasmic membrane by inducing membrane depolarization (Tokuda & Konisky, 1979). A similar conclusion was reached by Kayalar & Luria (1979) with colicin K.

In this paper we describe experiments to explore further the mode of action of colicin S8 on *Escherichia coli*. A first approach was reported by Nágel de Zwaig & Vitelli-Flores (1973). This bacteriocin is synthesized by a strain of *Shigella*, ADO3-8. Antón (1964) classified it following Fredericq's nomenclature as colicin S8. It is different from any of the other well characterized colicins because of the type of resistant mutants it selects. Strains resistant to

† Present address: Microbiology Unit, Department of Biochemistry, South Parks Road, Oxford OX1 3QU, U.K.

colicin S8 are also resistant to phage T6 (Antón, 1964). A similar characteristic is found with colicin K, since strains resistant to this colicin are also resistant to phage T6.

METHODS

Bacteria. The colicin was prepared from *Escherichia coli* K12 Row BA56 (Antón, 1964). *Escherichia coli* VE673, selected as a λ -resistant colony of *Escherichia coli* K12 C600 C10 (Nágel de Zwaig, 1969), was used as an indicator strain. *Escherichia coli* K12 3000Y14 HfrH (Nágel de Zwaig & Vitelli-Flores, 1973) was used for the experiments on the incorporation of radioactively labelled precursors.

Growth of bacteria. L-broth (Kennedy, 1971) was used except in experiments on the incorporation of radioactively labelled precursors when minimal glucose medium supplemented with thiamin ($5 \mu\text{g ml}^{-1}$) was used (Davis & Mingioli, 1950). Growth was followed by measuring either the absorbance at 520 nm in a 1 cm path-length cuvette in a Bausch & Lomb photocolormeter or the absorbance in a Klett photocolormeter fitted with a green filter. Inocula from overnight cultures were diluted 1:20 into fresh medium and grown at 37°C to the exponential phase before each experiment.

Preparation of colicin extracts. Colicin production was induced in two ways: (i) by adding mitomycin C ($6.4 \mu\text{g ml}^{-1}$) to a culture growing at 37°C and continuing growth in darkness for 3 to 5 h; or (ii) by irradiation of bacteria growing exponentially, and subsequent centrifugation and resuspension of the pellet in 3 to 5 ml of 0.1 M-phosphate buffer, pH 7, followed by growth at 37°C in darkness for 3 to 5 h. After induction two different kinds of extract were prepared. For supernatant extracts (S-extract), cultures were centrifuged, the supernatant was sterilized with chloroform and the colicin concentration was determined. S-extracts remained active at 4°C for longer than 1 month. To prepare cytoplasmic extracts (C-extract), bacterial pellets were resuspended in 0.1 M-phosphate buffer, pH 7, and sonicated for three intervals of 60 s using a MSE probe sonicator; in this way, the absorbance was reduced to 10%. Cell debris was separated by centrifugation at 5000 g for 15 min and the supernatant was sterilized with chloroform. Frozen C-extracts remained active for several months. S-extracts and C-extracts behaved indistinguishably in the experiments reported in this paper.

In vivo colicin assays. (i) *Stab test.* Colicinogenic strains were stabbed with a needle into L-broth agar. After overnight incubation at 37°C , the plates were exposed to chloroform vapour for 5 min, then a sensitive strain, grown overnight and suspended in soft agar, was overlaid. A clear 'halo' developed following incubation for 15 h, the size of which was dependent on the amount of colicin produced. (ii) *Qualitative titre.* Colicin extracts were diluted into 0.9% (w/v) NaCl. From each dilution 50 μl was spotted on L-broth plates previously overlaid with *E. coli* (strain VE673) suspended in L-broth soft agar. The plates were incubated for 14 h at 37°C and the colicin S8 titre (number of killing units ml^{-1}) was arbitrarily defined as the highest dilution that still gave a clear inhibition zone. (iii) *Quantitative titre.* Colicin extracts were diluted several times into 0.9% (w/v) NaCl. From each dilution 0.1 ml was taken and incubated for 10 min with 0.4 ml of exponentially growing *E. coli* (strain VE673). Samples were resuspended in 2.5 ml of soft L-broth agar and overlaid on L-broth plates. A control plate was prepared with 0.4 ml of culture and 0.1 ml of 0.9% (w/v) NaCl. The plates were incubated for 14 to 16 h at 37°C and colonies were counted. The titre [lethal units (L.U.) ml^{-1}] was calculated from the equation of Poisson: $B/B_0 = A \cdot e^{-x}$, where A is a constant, B_0 is the number of viable cells, B is the number surviving colicin treatment and x is the multiplicity (number of lethal units per bacterium). By taking the proportion of survivors after colicin treatment as being the proportion of cells which have not adsorbed a lethal unit, and using the Poisson distribution, the average number of lethal units per bacterium was calculated and the titre of a colicin solution was expressed in L.U. ml^{-1} (Reeves, 1972).

Titration with sodium dodecyl sulphate (SDS). A culture of strain VE673 was grown to an A_{520} of 0.5 at 37°C in L-broth supplemented with 0.2% (w/v) glucose and kept on ice. A series of dilutions of the colicin extract was prepared in the same medium; 0.5 ml of each dilution was added to 4.5 ml of the culture (A_{520} 0.5) and incubated for 10 min at 37°C . SDS (final concentration 0.5 mg ml^{-1}) was then added and the A_{520} was measured at 1 min intervals for 10 min.

Trypsin treatment. Bacteria were grown to the exponential phase and samples of 0.4 ml were incubated with 0.1 ml of colicin S8 extracts at 37°C . At intervals, 0.05 ml samples were taken and incubated with trypsin (0.25 mg ml^{-1}) in L-broth for 30 min at 37°C . Dilutions were then prepared in buffer at 0°C . Samples were titrated immediately (by the quantitative method). Samples without trypsin were titrated at each time interval from a control test tube.

Bacteriophage T4 experiments. Cells were grown to the exponential phase and bacteriophage T4 (about 5 phage particles per bacterium) was added at 0 min and again at 10 min. Growth was continued at 37°C with shaking and colicin S8 (multiplicity 2) was added. The absorbance of the culture was measured at 5 min intervals. To measure the effect of colicin S8 on phage reproduction, cells were treated with chloramphenicol (CAM; 50 $\mu\text{g ml}^{-1}$) and incubated with T4 (about 5 phage particles per bacterium) at 37°C for 4 min. After incubation, non-adsorbed phage was removed by centrifugation for 10 min at 10000 rev. min^{-1} in a Sorval RC2B centrifuge.

Bacteria were resuspended in pre-warmed medium and colicin extract at different dilutions was added. The culture was incubated at 37 °C for 10 min and the survival rate was determined. As a control, bacteriophage T4 reproduction was checked by determining the number of plaques produced at each dilution of colicin. The amount of non-adsorbed phage was <5% for each experiment.

Incorporation of radioactive precursors. Nucleic acid and protein synthesis in colicin S8-treated cells was assayed by the incorporation of [¹⁴C]uracil and [¹⁴C]isoleucine. After incubation of the cells with colicin at room temperature, [¹⁴C]uracil or [¹⁴C]isoleucine (each 20 µM; 0.1 µCi ml⁻¹, 3.7 kBq ml⁻¹) was added. At intervals, samples (0.5 ml) were taken and mixed with an equal volume of ice-cold 10% (w/v) trichloroacetic acid (TCA). After 30 min, these samples were filtered on Millipore filters (0.45 µm pore size) and washed twice with ice-cold 5% (w/v) TCA. The filters were dried, stuck on metallic planchets and counted in a spectrometer (Nuclear Chicago, model 720).

Chemicals. All chemicals used were the best grade commercially available. Mitomycin C and trypsin were obtained from Sigma and radiochemicals from Nuclear Chicago.

RESULTS AND DISCUSSION

Effect of trypsin on colicin S8-induced killing

Stage I of the action of colicin on bacteria has been defined operationally as the stage during which the colicin is susceptible to inactivation by trypsin (Plate & Luria, 1972). This stage is not an energy-requiring step. Trypsin partially prevented the lethal effect of colicin S8 in a way which was very similar to its action on colicin A. Dinitrophenol had no effect on trypsin action in this experiment. It was found that for only 8% of the population did colicin S8 added at 10 L.U. per cell remain accessible to trypsin (0.25 mg ml⁻¹) for at least 30 min at 37 °C. For more than 90% of the bacteria, the colicin S8 was not accessible to trypsin even after 2 min incubation. This could be the result of the binding itself or could be due to consequent molecular rearrangements in the surface structure. The latter is the more likely explanation since after colicin S8 treatment the bacteria become much more sensitive to lysis by SDS and have altered permeabilities (Nágel de Zwaig & Vitelli-Flores, 1973). Colicin S8 is thus similar to colicins E1, K and A and by analogy would be expected to alter the phospholipid composition of the membrane (Cavard *et al.*, 1974). This conclusion is supported by the observation of Nágel de Zwaig & Vitelli-Flores (1973) that the effect of colicin S8 on isoleucine uptake or incorporation is notably reduced when cells are transferred to a lower temperature which would alter the composition of the membrane lipid (Cramer *et al.*, 1973). Thus, the fluidity of the membrane is important for the colicin S8 action.

Lysis by SDS of bacteria pretreated with colicin S8

SDS acts as a detergent on the lipid-protein interactions of membranes (Cavard *et al.*, 1967). Bacteria such as *E. coli* B and *E. coli* K12 become sensitive to the action of SDS after various treatments, e.g. inhibition of oxidative phosphorylation, treatment with lysozyme plus EDTA, or with colicins like K and E1. Cavard *et al.* (1974) have shown that SDS at 0.5 mg ml⁻¹ reveals structural modifications of the cell membrane.

Bacteria pretreated with colicin S8 started to lyse about 3 to 5 min after the addition of SDS (Fig. 1) and there was rapid lysis during the next few minutes. Preincubation with CAM had little effect on the time at which lysis of a colicin S8-treated culture began but the lysis was much less rapid. Both CAM alone and CAM plus SDS caused some lysis but much less than when colicin S8 was present.

Thus, the synthesis of protein may be involved in the action of colicin S8. Mindich (1970) showed that in a glycerol-requiring mutant of *B. subtilis* CAM affected the synthesis of lipids, so that it is possible that the effect of CAM on colicin S8 action may be the result of changes in the lipid composition. Faster growing bacteria were found to be more susceptible to lysis by SDS following colicin S8 treatment; therefore metabolic reactions are necessary for the colicin effect on the cytoplasmic membrane.

Fig. 1. Lysis by SDS of bacteria pretreated with colicin S8. Samples of exponentially growing bacteria were removed to room temperature, and some were incubated with CAM ($50 \mu\text{g ml}^{-1}$) for 10 min. All samples were then transferred to 37°C , and some were incubated with colicin S8 (about 20 L.U. ml^{-1}) for 10 min. At this time (time 0), SDS (final concentration 0.5 mg ml^{-1}) was added to some of the samples, and the subsequent changes in absorbance of all samples were followed: $\square$, CAM; $\circ$, no additions; $\triangle$, CAM + SDS; $\blacksquare$, colicin S8 + SDS; $\bullet$, CAM + colicin S8 + SDS.

Fig. 2. Dependence of the rate of lysis on the colicin concentration. Samples of exponentially growing bacteria (1×10^9 bacteria ml^{-1}) were incubated at 37°C with various concentrations of colicin S8. After 10 min, SDS was added (final concentration 0.5 mg ml^{-1}) and the absorbance was measured at 1 min intervals for 10 min. A slope was extrapolated for each concentration on a graph of change in A_{520} against time, after lysis by SDS of colicin-treated cells had started. The values are the mean of at least two experiments (in most cases, three). The bars represent the standard deviation. A different preparation of colicin S8 used in a similar experiment gave essentially the same result.

Kinetics of lysis by SDS and a quick method of titration for colicin S8 preparations

Bacteria were treated with colicin S8 at several different concentrations before lysis with SDS. After a delay of 2 to 3 min, lysis occurred at a rate dependent on the colicin S8 concentration. The experiment was repeated several times and the mean rate of lysis was determined at various concentrations. A plot of the rate of lysis against concentration showed first-order kinetics (Fig. 2). The plateau at high concentrations was due to saturation of the bacterial receptors with colicin S8; about 0.3 L.U. per bacterium was sufficient to saturate the receptors on strain VE673.

Based on the above experiments a method was developed for the titration of colicin S8 in liquid medium. First an 'arbitrary lethal unit' was defined as the dilution of colicin S8 which will cause a change in A_{520} of $5 \times 10^{-2} \text{ min}^{-1}$ under the conditions of assay used in the

Fig. 3. Effect of colicin S8 on bacteria superinfected with bacteriophage T4. A culture of exponentially growing bacteria was divided into three samples and incubation was continued at 37 °C. To one sample, colicin S8 (about 1×10^{10} L.U. ml^{-1}) was added (○); T4 (5 phage per bacterium) was added to the other two samples and a second lot of T4 (5 phage per bacterium) was added 10 min later. After a further 20 min incubation, colicin S8 (about 1×10^{10} L.U. ml^{-1}) was added to one infected culture (●) and the other served as a control (□).

experiment reported in Fig. 2. The concentration of colicin is expressed as the reciprocal of this dilution.

This method of titration has proved to be useful because a quick result can be obtained. The titres calculated should give a better estimate of the actual number of killing units in the preparation, since some colicins seem able to attack a second cell after leaving a first one, so the actual number of killing units can be overestimated by measuring survival (Cavard *et al.*, 1971). A similar method was used to determine the titre of T4 ghost preparations (Cavard *et al.*, 1974) and also colicin K (Cavard, 1976).

Effect of colicin S8 on motility

Motility is dependent on aerobic metabolism so that those colicins that act on energy-related processes would be expected to inhibit motility (Fields & Luria, 1969). A drop (3×10^{10} L.U.) of a colicin S8 preparation was mixed with a drop of aerobically grown bacteria and the motility was observed by phase-contrast microscopy. After 3 min the bacteria were much less motile but had not completely stopped. In a control, colicin A (3×10^{10} L.U.) completely stopped movement within 3 min of addition.

Effect of T4 infection on susceptibility to colicin S8

Mukai *et al.* (1967) found that in bacteria infected with bacteriophage T4, superinfection with the same phage prevents the inhibition of metabolism and subsequent lysis caused by the primary infection. Substances such as potassium cyanide or anaerobic conditions provoke lysis of the superinfected bacteria. Bacteria superinfected with T4 were found to be more susceptible to lysis by colicin S8 (Fig. 3).

Nomura (1963) showed that colicin E2 does not affect the replication of bacteriophage T4 but that colicin K inhibits phage replication. He assayed the effect of colicins on the number of lytic plaques when a culture of susceptible *E. coli* cells were infected with T4 phage. In a similar experiment, we found that colicin S8 behaves similarly to colicin K in preventing T4 replication. Here again an effect on metabolism is suggested, but it is not possible to conclude whether this is a primary effect of the colicin. Permeability changes at the cell membrane with a subsequent effect on metabolic reactions could also be an explanation for this result.

Fig. 4. Multiplicity-dependent effect of colicin S8 on [^{14}C]isoleucine or [^{14}C]uracil incorporation. Cells were grown to the exponential phase in minimal glucose medium, chilled, centrifuged and resuspended in fresh minimal glucose medium. Colicin S8, at several dilutions, was added to 5 ml of the resuspended cells 10 min before the addition (at time 0) of the radioactive compound. Survival and multiplicity were determined 9.5 min after the addition of colicin. (a) Effect on isoleucine incorporation at 36 °C: ■, multiplicity 100, % survival 0.036; ●, multiplicity 10, % survival 0.3; △, multiplicity 5, % survival 0.85; □, multiplicity 1, % survival 6.07; ○, control. (b) Effect on uracil incorporation at 30 °C: ■, multiplicity 10, % survival 0.1; ●, multiplicity 5, % survival 2; □, multiplicity 2, % survival 4; ○, control.

Multiplicity-dependent effect of colicin S8 on isoleucine and uracil incorporation

We found that, even with multiplicities of colicin which are able to kill more than 90% of the cells and to produce maximum lysis by SDS, incorporation of isoleucine or uracil was never completely inhibited. Instead, a multiplicity-dependent effect was observed (Fig. 4a, b). In contrast, Fields & Luria (1969) found complete inhibition of RNA and protein synthesis by colicins E1 and K at lower concentrations than we used (1% survival with colicin K; 0.3% survival with colicin E1). The fact that we never found complete inhibition, even at very high multiplicities, could be due to ATP production from glycolysis in the cells grown in minimal medium containing glucose. If that is the case, colicin S8 does not affect glycolysis and is therefore different in its action from K and E1 (Fields & Luria, 1969). One possible interpretation for this multiplicity-dependent effect of colicin S8 could be mechanical disturbance of the cell surface organization due to colicin attached to ineffective wall or membrane receptors (Smarda, 1975). The more colicin molecules reaching the cell surface, the greater would be the secondary damage produced on functions associated with the cell surface. Recently, reversal by trypsin of the inhibition of active transport by colicin E1 was observed by Dankert *et al.* (1980).

In spite of all the similarities between the effects of colicins K, E1, A and S8, we think that they have different mechanisms of action that depend on the specific sites on the cell membrane upon which they act to affect a specific source of energy such as ATPase, cytochromes, proton pump, etc. (Harold, 1977). Understanding of the mechanisms should be achieved by looking at their effects on the whole cells along with those of membrane vesicles and cell walls. Recent work with colicin E1 supports this point of view. Uptake of colicin E1 is a complex membrane-dependent transport system involving the interaction of numerous cell surface components (Kadner & McElhaney, 1980).

We are most grateful to Dr K. G. H. Dyke for critical advice in the preparation of the manuscript. We also thank Mr D. Ramsay for help with the illustrations. This investigation was partially supported by the Consejo de Desarrollo Científico y Humanístico de la Universidad de Los Andes.

REFERENCES

- AUTÓN, D. N. (1964). Thesis, Universidad de Buenos Aires, Argentina.
- BOWMAN, C. M., DAHLBERG, J. E., IKEMURA, T., KONISKY, J. & NOMURA, M. (1971). Specific inactivation of 16S ribosomal RNA induced by colicin E3 *in vivo*. *Proceedings of the National Academy of Sciences of the United States of America* **68**, 964–968.
- BUXTON, R. S. & HOLLAND, I. B. (1974). Colicin E2 induced DNA solubilization in a mutant of *E. coli* deficient in endonuclease I. *FEBS Letters* **39**, 1–3.
- CAVARD, D. (1976). Sensitization of colicin K-treated bacteria by sodium dodecyl sulphate: presence of free colicin in colicin K-treated cultures of *E. coli*. *Antimicrobial Agents and Chemotherapy* **9**, 639–645.
- CAVARD, D., POLONOVSKI, J. & BARBU, E. (1967). Modifications du métabolisme des phospholipides de *E. coli*, consécutives à l'action de la colicine K. *Compte rendu hebdomadaire des séances de l'Académie des sciences* **D265**, 1851–1854.
- CAVARD, D., MAROTEL-SCHIRMANN, J. & BARBU, E. (1971). Reversion de la fixation des colicines. *Compte rendu hebdomadaire des séances de l'Académie des sciences* **D273**, 1167–1170.
- CAVARD, D., VALLÉE, M. & BARBU, E. (1974). Mise en évidence par le LS de quelques modifications précoces de la membrane de *E. coli* provoquées par les phages T4, les fantômes de phages T4 et la colicine K. *Biochimie* **56**, 221–230.
- CRAMER, W. A., PHILLIPS, S. K. & KEENAN, T. W. (1973). On the role of membrane phase in the transmission mechanism of colicin E1. *Biochemistry* **12**, 1177–1181.
- DANKERT, J., HAMMOND, S. M. & CRAMER, W. A. (1980). Reversal by trypsin of the inhibition of active transport by colicin E1. *Journal of Bacteriology* **143**, 594–602.
- DAVIS, B. D. & MINGIOLI, E. S. (1950). Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *Journal of Bacteriology* **60**, 17–28.
- FIELDS, K. L. & LURIA, S. E. (1969). Effects of colicins E1 and K on cellular metabolism. *Journal of Bacteriology* **97**, 64–77.
- HAROLD, F. M. (1977). Membranes and energy transduction in bacteria. *Current Topics in Bioenergetics* **6**, 83–149.
- KADNER, R. J. & MCELHANEY, J. (1980). Other membrane-dependent transport systems in *Escherichia coli*. Effect of repression or cessation of colicin receptor synthesis on colicin receptor activities. *Journal of Bacteriology* **143**, 135–141.
- KADNER, R. J., BASSFORD, P. J., JR & PUGSLEY, A. P. (1979). Colicin receptors and the mechanisms of colicin uptake. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene (I Abteilung, Originale A)* **244**, 90–104.
- KAYALAR, C. & LURIA, S. E. (1979). Channel formation by colicin K on liposomes. *Bacteriological Proceedings* **6**, 297–306.
- KENNEDY, C. K. (1971). Induction of colicin production by high temperature of inhibition of protein synthesis. *Journal of Bacteriology* **108**, 10–19.
- MINDICH, K. (1970). Membrane synthesis in *Bacillus subtilis*. II. Integration of membrane proteins in the absence of lipid synthesis. *Journal of Molecular Biology* **49**, 433–439.
- MUKAI, F., STREISINGER, G. & MILLER, B. (1967). The mechanism of lysis in phage T4-infected cells. *Virology* **33**, 398–404.
- NÄGEL DE ZWAIG, R. (1969). Mode of action of colicin A. *Journal of Bacteriology* **99**, 913–914.
- NÄGEL DE ZWAIG, R. & VITELLI-FLORES, J. (1973). Mode of action of a new colicin. *FEBS Letters* **29**, 318–322.
- NOMURA, M. (1963). Mode of action of colicines. *Cold Spring Harbor Symposia on Quantitative Biology* **28**, 315–324.
- PLATE, C. A. & LURIA, S. E. (1972). Stages in colicin K action, as revealed by the action of trypsin. *Proceedings of the National Academy of Sciences of the United States of America* **69**, 2030–2034.
- REEVES, P. (1972). *The Bacteriocins*. Berlin: Springer-Verlag.
- SMARDA, J. (1975). Novel approaches to the mode of action of colicins. *Folia microbiologica* **20**, 264–271.
- TOKUDA, H. & KONISKY, J. (1979). Effect of colicins Ia and E1 on ion permeability of liposomes. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 6167–6171.