Arch Microbiol (1989) 151:486–490

Archives of
Microbiology
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Identification of aggregation substances of *Enterococcus faecalis* cells after induction by sex pheromones

An immunological and ultrastructural investigation

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Abstract. The sex pheromone system of *Enterococcus faecalis* is responsible for the clumping response of a plasmid carrying donor strain with a corresponding plasmid free recipient strain due to the production of sex pheromones by the recipient strain. The clumping response is mediated by a surface material (called aggregation substance) which is synthesized upon addition of sex pheromones to the cultures. Here we show that after induction a dense layer of "hairlike" structures is formed on the cell wall of the bacteria. These hairlike structures are responsible for the cell-cell contact which leads to the aggregation of cells. Formation of these structures was specific, only occurring after the addition of homologous sex pheromone.

Key words: Aggregation substance – *Enterococcus faecalis* – Sex pheromone system – Transmission electron microscopy – Immunogold labelling technique

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The sex pheromone system of *Enterococcus faecalis* was discovered and first described by the group of D. B. Clewell (Dunny et al. 1978) and is characterized by a plasmid exchange mechanism. A model for the function of the sex pheromone system was first formulated by Ike and Clewell (1984) whereby plasmid free strains of *E. faecalis* excrete small linear peptides which act specifically on strains carrying a corresponding plasmid. In response to the presence of sex pheromones the plasmid carrying donor strain synthesizes a "sticky material", located on the cell surface, which is of a proteinaceous nature (Yagi et al. 1983; Kessler and Yagi 1983; Tortorello et al. 1986; Ehrenfeld et al. 1986; Ehrenfeld and Clewell 1987). This so-called "aggregation substance" enables the donor strain to aggregate with the plasmid free recipient strain. As a consequence of this "clumping" the corresponding plasmid is transferred via conjugation to the recipient strain. Conjugation frequencies may be several orders of magnitude higher for plasmids transferred via the sex pheromone system than for conjugative plasmids which do not respond to sex pheromones. Since the donor strain possesses a mechanism which shuts off production of the sex pheromone compatible with the donor's plasmid, self-clumping is not observed in nature. However, such a self-clumping can be induced in vitro by the addition of the corresponding sex pheromone to a donor strain. Donor cells are capable of producing all other sex pheromones not corresponding to the plasmid(s) they carry. The chemical structure of some sex pheromones has been recently determined (Mori et al. 1986); they were found to be small, linear, very hydrophobic peptides consisting of seven (in two cases) or eight amino acids (in two other cases). No unusual amino acids could be detected. Chemically synthesized sex pheromones were shown to be as effective as the natural peptides. Sex pheromones are named according to their respective sex pheromone plasmid: e.g. the sex pheromone cAD1 (c standing for clumping) induces formation of an aggregation substance in a pAD1 (p standing for plasmid) carrying donor strain.

Yagi et al. (1983) and Kessler and Yagi (1983) have shown that the sticky material synthesized by donor strains in response to sex pheromones may act as an adhesin. They postulated that the aggregation substance is distributed equally over the cell surface, however, they were unable to demonstrate directly the existance of an aggregation substance. Using transmission electron microscopy we demonstrate here for the first time the presence of an aggregation substance in situ on the cell surface.

Materials and methods

Bacterial strains and media

The strains used in this study are listed in Table 1. They were grown in antibiotic medium 3 (Oxoid) at 37°C with mild agitation. Chemically synthesized sex pheromones (gift of D.B. Clewell), used to induce formation of the aggregation substance, were added at an optical density of 0.025 (measured at 580 nm).

Purification of the aggregation substance

The aggregation substance was purified from strain FA2-2:pAM721. This strain is a constitutive producer of aggregation substance due to an insertion of transposon Tn917 in its plasmid pAD1 (Ike and Clewell 1984). Cells in midexponential growth phase (ca. 400 OD units) were harvested by centrifugation (15 min at 6000 × *g*), washed

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Abbreviations: cAD1, sex pheromone specific for plasmid pAD1; cPD1, sex pheromone specific for plasmid pPD1; CW, cell wall; pAD1, conjugative plasmid specifically transferred in the presence of cAD1; pPD1, conjugative plasmid specifically transferred in the presence of cPD1; PBS, 10 mM Na-phosphate pH 7.5, 0.85% NaCl

Table 1. *Enterococcus faecalis* strains tested for presence of aggregation substance after induction with sex pheromones

Strain	Relevant genotype	Reference
FA2-2	plasmid-free recipient	Clewell et al. (1982)
OG1X	plasmid-free recipient	Ike et al. (1983)
OG1X:pAD1	carries sex pheromone plasmid pAD1; donor strain	this study
OG1X:pPD1	carries sex pheromone plasmid pPD1; donor strain	this study
OG1S:pPD1	carries sex pheromone plasmid pPD1; donor strain	Franke and Clewell (1981)
DS16:pAD1	carries sex pheromone plasmid pAD1; donor strain	Franke and Clewell (1981)
FA2-2:pAM721	carries sex pheromone plasmid pAD1 with a Tn917 insertion; donor strain	Ike and Clewell (1983)

twice in 50 ml PBS and resuspended in 4 ml PBS containing 0.2% "Zwittergent 3-12" (Calbiochem, Los Angeles, CA, USA). The aggregation substance was extracted from 1 ml portions by shaking for 60 min in a 1.5 ml Eppendorf reaction vial in an Eppendorf 5432 mixer. The extracts were separated from cells by centrifugation (15 min at $13000 \times g$) and dialyzed overnight at 4°C against bidistilled water. These samples were lyophilized and aliquots tested for purity on standard SDS 8% acrylamide gels (Laemmli 1970). Extracts of more than 95%purity were used for immunizations and direct electron microscopic examinations.

Preparation of antiserum and indirect immunogold-labelling

Antiserum was raised in female rabbits (chinchilla-hydrids, Thomae, Biberach, FRG) using an immunization scheme described by Hennecke et al. (1977). To eliminate possible unspecific antibodies the crude antiserum was incubated overnight at 4°C with uninduced cells plus cell-lysate of strain DS16:pAD1; thereafter cells were removed by centrifugation (15 min at $13000 \times g$) and the supernatant filter-sterilized. The IgG fraction was then purified from the antiserum by protein G-Sepharose column chromatography as described by the supplier (Pharmacia, Freiburg, FRG).

For indirect immunogold labelling experiments the highly specific antiserum prepared against purified aggregation substance was incubated with the cells to be tested. The second step involved incubation of the primary immunoconjugate with "Auro Probe EM" (goat-anti-rabbit antibodies coupled to colloidal gold with a mean particle size of 9.5 nm) from Janssen Biotech N.V. (Olen, Belgium). The detailed protocol for indirect immunogold labelling was as follows: cells corresponding to 2 optical units (measured at 580 nm) were washed with 1 ml of PBS containing 1 mM Na-azide, and resuspended in 1 ml PBS containing 20 μl (ca. 70 μg) of the purified IgG fraction directed against aggregation substance. The cell/antibody suspension was incubated for 2 h at room-temperature with occasional mixing and then washed twice with 1.5 ml PBS. The cells were resuspended in 1 ml PBS plus 0.35 ml of "Auro Probe EM". Incubation was for 3 h at room-temperature with occasional mixing. After two washing steps in PBS the labelled cells were fixed as described below.

Western-blot analyses

Immunoblot analyses were performed using horse-radish-peroxidase coupled to goat-anti-rabbit IgG antibodies (Sigma, St. Louis, MO, USA). The proteins were separated on standard SDS-acrylamide gels (Laemmli 1970), transferred onto nylon membranes (Nytran from Schleicher & Schüll, Dassel, FRG) and detected as recommended by the producer.

Electron microscopy

Freshly harvested cells were fixed for 60 min at room temperature with 2.5% glutardialdehyde in 75 mM sodium cacodylate, 1 mM $MgCl_2$, pH 7.2. After the cells had been rinsed with the same buffer, they were postfixed for 30 min in 1% osmium tetroxide in buffer. For en bloc staining the cells were incubated with 1% uranyl acetate in 20% acetone for 30 min. Dehydration was with a graded series of acetone solutions. Cells were then infiltrated and embedded in Spurrs low viscosity resin (Spurr 1969). After polymerization the resin was cut with a diamond knife on a Reichert-Jung Ultracut E Ultramicrotome and the sections mounted on uncoated or collodion coated copper grids. The sections were post-stained with aqueous lead citrate (3%, pH 13). All pictures were taken with an Elmiscop 101 (Siemens) electron microscope.

Results and discussion

After addition of the sex pheromones (homologous induction) clumping of cells could be observed both macroscopically and with the aid of a light microscope. In the case of non-homologous "inductions", clumps were observed neither macroscopically nor by light microscopy.

Electronmicroscopic examination of induced and uninduced cells showed a clear ultrastructural change of the cell surface. Ultrathin sections of non-induced or non-homologous "induced" cells exhibited a smooth surface (Fig. 1). However, the cell surface of induced cells was coated with hairlike structures which are embedded in, and protrude from the cell wall ca. 18 nm (Fig. 2). Longitudinally, the hairs often exhibited a dark-light-dark-light pattern: (3 nm/5 nm/3 nm/5 nm) which suggests that they are tubular in structure (Fig. 2). Examination of tangentially sectioned cell surfaces support the idea of the tubular nature of these structures (Fig. 2). Neither cross nor longitudinal sections allowed a clear delineation as to whether the core of these structures is the dark or the light component (Fig. 2, dark arrows). Since these structures appear in some cases to penetrate the cell wall (thickness 15 nm; see Fig. 2, open arrow) we tentatively hypothesize that they are tubular, ca. 13 nm wide and 33 nm long.

Electron microscopic investigation of isolated aggregation substance is difficult because of its inherent stickiness, thus clumping together. However, negatively stained preparations show clusters of ringlike structures approx. 14 nm in diameter (Fig. 3) which supports the idea of the tubular nature of the aggregation substance. Clearly, an improve-

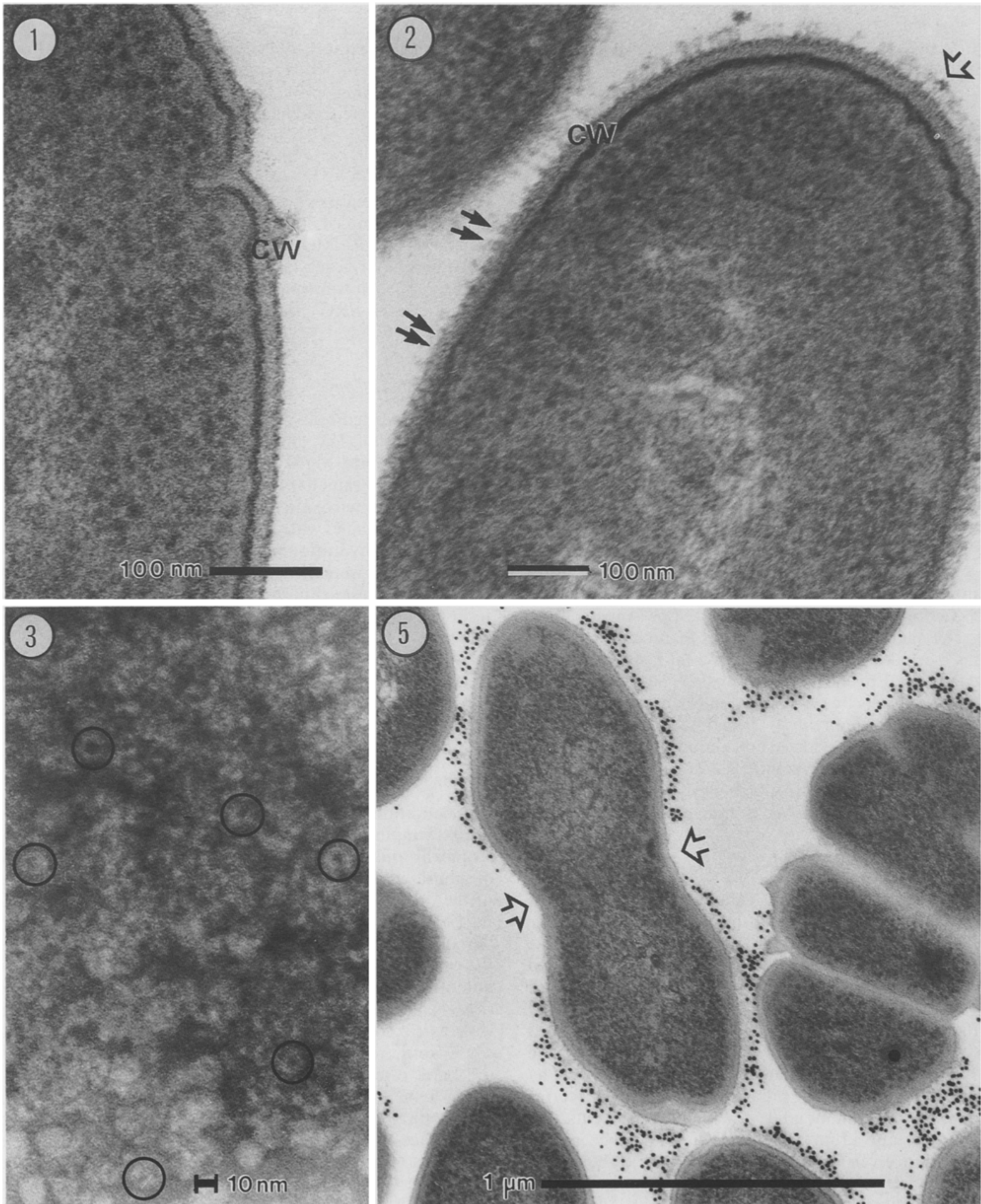

Fig. 1. Transmission electron micrograph of ultrathin section of a non-homologously "induced" *Enterococcus faecalis* strain (OG1X:pPD1 + cAD1). The cell wall architecture is identical in appearance to the uninduced control (OG1X:pPD1)

Fig. 2. Cells of *Enterococcus faecalis* induced by homologous sex pheromone (OG1X:pAD1 + cAD1) showing the "hairlike structures" which mediate the clumping response (*open arrow*). Apparent "breaks" in the cell wall (*open arrow*) indicate that these structures penetrate into the cell wall. Tangential sections show evidence for the possible tube-like nature of these structures (*dark arrows*)

Fig. 3. Isolated and purified aggregation substance ("hairlike structures") showing ring-like structures ca. 14 nm in diameter (*circles*) after negative staining with potassium phosphotungstate. As expected even in its purified form is has an intense aggregation quality

Fig. 5. Ultrathin section of induced and immunogold labelled *Enterococcus faecalis* cells (OG1X:pAD1 + cAD1) showing that the immunogold label attaches exclusively to the "hairlike" aggregation substance (see Fig. 2); note the absence of immunogold label at the septation area (*open arrows*)

Table 2. Correlation of clumping in culture and the appearance of "hairlike structures" for various *Enterococcus faecalis* strains

Bacterial strains	Sex pheromones[a]	Clumping	"Hairlike structure"
DS16:AD1	–	–	–
DS16:pAD1	+ cAD1	+	+
DS16:pAD1	+ cPD1	–	–
OG1S:pPD1	+ cAD1	–	–
OG1S:pPD1	+ cPD1	+	+
OG1X:pAD1	+ cAD1	+	+
OG1X:pPD1	+ cAD1	–	–
FA2-2:pAM721	–	+	+
DS16:pAD1	+ FA2-2	+	+

[a] cAD1 and cPD1 denote synthetic sex pheromones specific for the plasmids pAD1 and pPD1; FA2-2 denotes that as a natural source of sex pheromone cAD1 cells in their growth medium were used

ment of investigative techniques will be required to allow more detailed analyses of these structures.

The aggregation substance was identified by the following approaches:

1. Comparisons of differently induced cells: uninduced cells of *E. faecalis* FA2-2, OG1X, OG1X:pAD1 and OG1X:pPD1 never showed the hairlike structures typical of the aggregation substance. After non-homologous inductions of the donor strains – i.e. addition of synthetic sex pheromone cPD1 to strain OG1X:pAD1 and of synthetic sex pheromene cAD1 to strain OG1X:pPD1 – these structures were also absent. Table 2 shows the correlation between the presence of the hairlike structures and the clumping of the cells.

2. Comparisons of cell aggregates: ultrathin sections of aggregates obtained by co-culturing donor and recipient strains (DS16:pAD1 and FA2-2, respectively) showed that the hairs protrude from one partner in the aggregates to the other, but not mutually between both cells. This picture was different for donor-donor aggregates: here indeed the hairs form a zone of contact protruding mutually from both cells (Fig. 2).

3. Immunological identification: an antiserum which specifically inhibited the in vivo aggregation response, and which was directed against almost pure aggregation substance reacted specifically with the observed hairlike structures. The protein was purified from strain FA2-2:pAM721 as outlined above. The specificity of the antiserum was tested by reacting it with cell wall extracts of cAD1 induced, as well as with non-induced *E. faecalis* strains OG1X:pAD1, DS16:pAD1, and OG1X:pPD1, and with cell wall extracts of strain FA2-2:pAM721 without induction. After transfer onto nylon membranes reaction was observed only for extracts from the strains OG1X:pAD1 and DS16:pAD1 both after homologous induction, and for the constitutive producer of the aggregation substance (strain FA2-2:pAM721) without induction. In each case this reaction was specific for a protein species of ca. 78 kD (see Fig. 4).

4. Immunogold labelling was specific for the induced hairlike structures: ultrathin sections of cells which were treated with the primary antiserum followed by the secondary antibody-gold complex, revealed that reaction occurred exclusively with the "hairs" induced by sex pheromone but not with uninduced cells (see Fig.5).

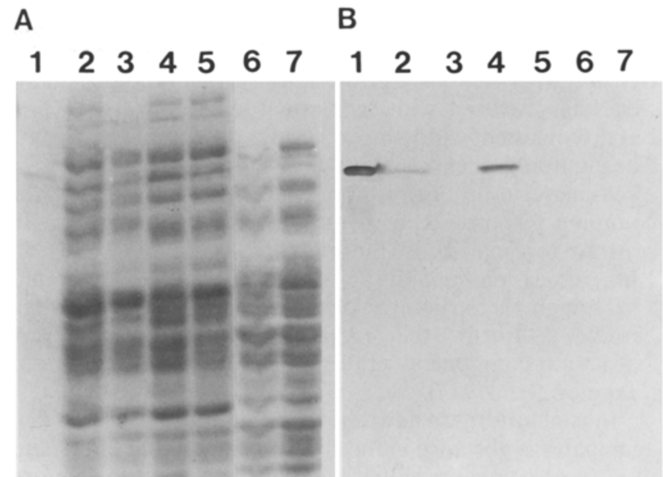

Fig. 4 A, B. Cell wall extracts of various *Enterococcus faecalis* strains after separation on SDS 8% acrylamide gel and Coomassie protein staining (**A**) or Western blot analysis (**B**). *1* FA2-2:pAM721; *2* DS16:pAD1 + cAD1; *3* DS16:pAD1; *4* OG1X:pAD1 + cAD1; *5* OG1X:pAD1; *6* OG1X:pPD1 + cAD1; *7* OG1X:pPD1. Protein staining shows that only in the case of FA2-2:pAM721 a preparation of almost pure aggregation substance can be obtained. Western blot analysis shows that only in the case of homologous cAD1 induction one protein reacts with antibodies directedly against preparation 1

The identification of aggregation substance was facilitated by correlating the clumping response with the appearance of the "hairs", and by using immunological methods. The fact that the antiserum detected only one protein, extractable from induced, but not from uninduced cells, indicated that the immunological method used for identification of the aggregation substance is highly specific. Strain FA2-2:pAM721 was chosen as the source for isolation of aggregation substance since this strain yielded the most reproducible results. The other strains showed varying degrees of lysis during the extraction procedure, resulting in preparations which were unsuitable for immunizations. This phenomenon is very clearly seen in Fig. 4: all samples loaded on the SDS-polyacrylamide gels were extracted identically.

Aggregation substance was shown earlier to be of proteinaceous nature (Yagi et al. 1983; Kessler and Yagi 1983). It was postulated subsequently that a major protein of 74 kD, together with minor proteins of 130, 150 and 157 kD, participated in the clumping reation of pAD1 carrying donor strains (Ehrenfeld et al. 1986). We have shown here, that an antiserum raised against a purified, pAD1 specific, 78 kD protein inhibits the clumping response of pAD1 carrying donor strains, but not of pPD1 carrying donor strains. This evidence strongly suggests that this protein is the major component of the pAD1 specific aggregation substance. We determined that the purified protein has a molecular weight of ca. 78 kD (data not shown); this value correlates well with the earlier observed major protein described by Ehrenfeld et al. (1986) The high-molecular weight proteins seen by these authors could scarcely be detected under our conditions. Therefore, it remains to be determined if these proteins might be precursors for aggregation substance or whether they are somehow involved in the aggregation event, in an "unspecific" manner, since they are observed not only in the pAD1 but also in the pPD1 system (see Ehrenfeld and Clewell 1987).

The hairlike structures demonstrated here resemble those described earlier as "fibrils" in certain strains of *Streptococcus salivarius* (Handley et al. 1984). These authors define fibrils as structures with "consistent length (76 to 289 nm) but no consistent width due to various degrees of clumping". The clumping phenomenon which these authors refer to is a self-aggregation of fibrils and, therefore, unrelated to the clumping response seen in the sex pheromone system. In contrast to such fibrils, fimbriae are "much longer (0.5 to 1 μm), consistent in width (3 to 4 nm) and do not "clump".

Though the structures observed here are shorter than Handley's "fibrils" they resemble them in that they are "structural components upon which adhesions are carried" (Handley et al. 1984).

In conclusion, we have shown here for the first time that aggregation substance induced by sex pheromones in strains of *E. faecalis* consists of hairlike structures which are located on the cell surface of the bacteria. The accompanying paper demonstrates that the distribution of these structures is dependent on the duration of induction by sex pheromones.

Acknowledgements. We wish to thank D.B. Clewell for strains and a generous gift of synthetic sex pheromones, A. Böck and H. Formanek for helpful discussions and H. Hirt for technical help. The technical assistance and editorial help of S. Schoy-Tribbensee, G. Sawers, and S. Reese is gratefully acknowledged. This work was supported by grants B8 and Y1 to RW and GW, respectively in the Sonderforschungsbereich 145 by the Deutsche Forschungsgemeinschaft. DG gratefully acknowledges the receipt of an educational grant by the University of München.

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Received November 30, 1988/Accepted January 25, 1989