Bacterial differentiation within *Moraxella bovis* colonies growing at the interface of the agar medium with the Petri dish

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Moraxella bovis was found to colonize the interface between agar and the polystyrene Petri dish, producing circular colonies when the inoculum was stabbed at a single point. The bacteria occurred in a thin layer of nearly uniform thickness, and colonial expansion occurred in at least two temporal phases. In the first phase, the radial colonial expansion was slow and non-linear. In the second phase, the radial expansion was linear. The interfacial colonies possessed three characteristic concentric growth zones. At the periphery was a narrow ring zone that enclosed another wider ring zone, which, in turn, surrounded a central circular zone. Different bacterial phase variants were recovered from these zones. The two outer ring zones yielded bacteria that formed agar surface colonies of spreading-corroding morphology, while cells from the innermost zone always yielded colonies with a different morphology. The uniform thickness of the colonies implied that replication was restricted to the outermost ring, and that the bacteria within the inner ring and inner circle had entered a quiescent state. The inner ring appeared to represent the lag in time needed for the replicative form to differentiate into the quiescent form. A different kind of variant was associated with wedge-shaped sectors within the colonies. The greatest number of these clonal variants appeared shortly after inoculation and their frequency decreased after the onset of linear growth. The period of slowest colonization coincided with highest frequency of clonal variant expression. It is proposed that the proliferative rate of the parental bacterial population exerted selective pressure on the expression of new clonal variants.

Introduction

While examining the growth on agar medium of *Moraxella bovis*, the causative agent of infectious bovine keratoconjunctivitis, circular halos were noticed beneath the agar where the bacteria had been stabbed. These halos were composed of bacteria and they only appeared if an agar-corroding form of the bacteria (Bøvre & Frøholm, 1972) was used as the inoculum. While colonies on the air surface never reached a size of more than a few millimetres in diameter, the colonies that formed beneath the agar would colonize the entire bottom of the Petri dish in only a few days. The interfacial colonies always had three concentric growth zones that could not be so readily distinguished in air surface colonies. These zones were discovered to have different phase variants associated with them. However, the spontaneous appearance of wedge-shaped sectors or clones originating at

various distances from the inoculation site represented a different mode of differentiation, and these sectors were similar to those seen in the air-surface colonies. Using the symmetry of these colonies, the incidence of both phase variants and clonal variants could be studied.

Bacterial variation, or differentiation, plays an important role in the survival of pathogenic bacterial species (Mekalanos, 1992), and there are at least two known mechanisms of bacterial differentiation. In response to environmental conditions such as changes in nutrient concentration or changes in temperature, bacteria can differentiate by gene regulation. Gene regulation has been demonstrated for the thermal regulation of gene expression by *Escherichia coli* (Göransson *et al.*, 1990), the expression of invasive capacity by *Salmonella* (Lee & Falkow, 1990), and mucoid conversion by *Pseudomonas aeruginosa* (Terry *et al.*, 1991). This form of differentiation is often reversible, and the bacteria expressing one or another of the forms are sometimes called phase variants. The second means by which bacteria differentiate is by rearranging or modifying a DNA sequence. Much of our current under-

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standing of this form of differentiation comes from studies of *Neisseria gonorrhoeae*, which can change its pilin and other outer surface components (Meyer & van Putten, 1989). It seems likely that both forms of differentiation occur in *M. bovis*, and provide this species with considerable flexibility for meeting environmental challenges.

Phase variants of *M. bovis* were first described by Bøvre & Frøholm (1972), who characterized them according to their distinctive colonial morphologies on agar media. Colonies with the spreading-corroding morphology are called the SC colonial form. Colonies with the non-spreading and non-corroding morphology are called the N colonial form, and a third form, with an intermediate morphology, is called the NSC colonial form. Primary isolates from bovine eyes are almost exclusively comprised of the SC colonial form (Pedersen, 1970; Pedersen *et al.*, 1972). The SC colonial form is also more virulent than the N colonial form when used to challenge the eyes of the host animal (Pedersen *et al.*, 1972; Jayappa & Lehr, 1986). Yet, shortly after the bacteria are recovered from infected cattle, the less-virulent N form begins to appear at high frequency and the virulent colonial form would be lost if it were not carefully selected for passage. In the present report, different colonial forms were found to be associated with the different growth zones of the interfacial colonies. This suggested that an additional function of phase variation might be related to proliferative state, and that specific environmental conditions may trigger differentiation of one replicative state into another.

The expression of pilin molecules with altered amino acid sequences by *M. bovis*, however, is the result of genotypic differentiation. Changes in the pilin molecule are a consequence of an inversion of a segment of DNA within the pilin gene (Marrs *et al.*, 1985). In the interfacial colonies, the spontaneity of sector appearance and the sector shape itself suggested unstable, genotypically controlled phenotypes as seen for other bacterial species. No attempt was made to confirm or reject a genetic origin of the sectors in this report. However, even if they were not of genetic origin, they may represent an important aspect of *M. bovis* pathogenicity. The sector variants attracted the author's attention because the circular shape of the colonies afforded an easy way of examining whether they had occurred randomly. Mutations within *E. coli* colonies do not appear randomly but in a brief 'burst' shortly after inoculation (Hall, 1988; Shapiro, 1984). This burst is typified by the appearance of only a few sectors immediately after inoculation, followed by the expression of many sectors. This, in turn, is followed by a decline in sector expression. A similar phenomenon was seen for the *M. bovis* interfacial colonies. In the *M. bovis*

colonies, however, it was possible to measure growth characteristics both preceding and during the clonal variant burst. This led to the realization that clonal variant expression may be linked to colonization rate.

The characteristics of *M. bovis*, when colonizing the interface between the agar and the Petri dish, suggested that incidence of the two different kinds of differentiation should be studied as a function of time. Most bacterial colonies approximate spherical segments in symmetry (Palumbo *et al.*, 1971; Wimpenny, 1979). This is probably due to the uneven diffusion of oxygen and nutrients (Reyrolle & Letellier, 1979; Fraleigh & Bungay, 1986). The *M. bovis* interfacial colonies, however, formed thin flat cylinders, or disks, only a few bacteria thick. This symmetry fits the model of colonial growth proposed by Pirt (1967, 1975). In his model, bacterial proliferation was restricted to the periphery of the colonial disk, and the thin uniform layer of bacteria in the interior of the colony stopped growing. The existence of this simple model fitting the growth characteristics of the interfacial *M. bovis* colonies provided the basis for examining the appearance of the different kinds of variants within the colonies.

Methods

Bacteria. Except when specified, the Med72(23R) isolate of *M. bovis* was used in all experiments. This and other isolates were kindly provided by Dr Kenneth Kopecky of the United States National Animal Disease Center, Ames, IA, USA. The morphology of colonies streaked on the agar-air surface was examined by stereomicroscopy (Bøvre & Frøholm, 1972). To inoculate the interface between the agar and the Petri dish bottom, a single agar-air colony was picked with an inoculation needle and plunged through the agar so that it touched the dish bottom at a single point. The needle was tapped lightly on the Petri bottom to ensure the success of the inoculation. The bacteria were grown at 35 °C, with 5% (v/v) carbon dioxide and 95% air, on Mueller-Hinton agar (Gibco) and on GC medium with defined supplements (ATCC no. 1074) in polystyrene Petri dishes (100 × 15 mm, diSPo Petri dish, American Scientific Products).

Pilus antiserum preparation. Pilus antigen was prepared from the Med72(23R) isolate by the method of Villela (1981). The bacteria were grown on the surface of Mueller-Hinton agar overnight at 35 °C and harvested into 10 mM-Tris/HCl buffer, pH 7.0. This suspension was blended for 2 min in a Waring blender and centrifuged at 4 °C for 30 min at 30000 g. Saturated ammonium sulphate solution was added to the supernatant to a final volume of 20% (v/v). Following centrifugation at 4 °C at 30000 g for 2 h, the pellet was retained and resuspended in 10 mM-Tris/HCl buffer. The pilus preparation was then recycled again in the same manner. SDS-PAGE of the pilus preparation resulted in a single band with a relative molecular mass of 20 kDa using both Coomassie brilliant blue R250 and silver staining.

New Zealand White rabbits were immunized with purified pilus preparations adsorbed to aluminium hydroxide (Rehsorptar II, Reheis Biochemical Co.). The protein content was determined using the Lowry method. Each rabbit was immunized three times, at intervals of 2 weeks, with 50 µg pilus protein per injection. Serum was collected 1 week after the final injection and stored at -36 °C.

Twitching motility. To observe twitching motility, the agar was removed from the dish, and a drop of normal saline placed on the bacteria remaining on the dish. A cover slip was placed on the drop, and then the dish placed on the stage of a microscope equipped with darkfield optics.

In situ staining of bacterial growth. The growth at the Petri-dish-agar interface was stained using two procedures. In the first, the agar was removed and the dish bottom dried under a heat lamp. The adherent bacteria were then stained with either Gram's stain or Coomassie blue. To stain the bacteria with Coomassie blue, the dried bacteria were flooded with 0.5% (w/v) Coomassie brilliant blue R250 dissolved in an aqueous solution containing 40% (v/v) methanol and 10% (v/v) acetic acid. Following incubation for 1 min, the excess stain was removed by several washes with an aqueous solution of 40% methanol and 10% acetic acid.

In the second method, the bacteria were grown under a thin agar layer (≤ 4 mm). The agar was left in the dish and a piece of round Whatman no. 1 filter paper was laid on the agar surface. Several layers of dry paper towels were then laid on the filter paper. To ensure the filter paper and towels had close contact with the agar, a weight (approximately 1 kg) was placed on top. The towels were changed every 10 min until most of the water had been pressed from the agar. When the agar was reduced to a thin film, the growth was stained with Coomassie blue and destained as described above. After destaining, the agar was dried under a heat lamp for permanent storage.

Nitrocellulose blotting of pili adhering to the agar. To detect piliated bacteria within the colonization area, the agar was removed intact and the colonies were blotted onto nitrocellulose by a procedure similar to the blotting of proteins from agarose (McMichael *et al.*, 1981). A nitrocellulose membrane was placed directly on the inverted agar after removal from the Petri dish. This was overlaid with several pieces of paper towelling, a flat plate and a 1 kg weight. After approximately 10 min, the nitrocellulose was immersed in a 5% (w/v) bovine serum albumin blocking solution and then probed with the rabbit anti-pilus sera. Bound rabbit antibodies were detected using ^{125}I -conjugated anti-rabbit immunoglobulins (New England Nuclear).

Colonization rate. The colonization rate was determined by measuring increase in colony diameter at different times during the colonization. To correct for slight variations in circularity, the diameter of each colony was taken as the mean of two transverse measurements. These diameters were then plotted against time. The rate of colonization was provided by the slope of this plot.

Determination of mean bacterial density. The average density of bacterial growth at the interface was determined by inoculating Petri dishes at a single site beneath the agar, so that circular zones of growth were obtained. As the colonies increased in size, plates were removed and both the area colonized and the total number of bacteria determined. The colonized area was determined from the mean of two transverse measurements of the diameter. To determine the number of bacteria that had colonized the interface, the bacteria growing at the air surface of the agar about the stab site were gently cut away. The agar and adherent bacteria was cut at a radius slightly larger than the outer edge of the growth and placed in a small flask. Residual bacteria adhering to the Petri dish were collected by washing the exposed area of the dish bottom once with 0.05% SDS, followed by several washes with phosphate-buffered saline (PBS; pH 7.0, 0.08 M-sodium phosphate, 0.12 M-NaCl). These washes were combined with the agar in the flask. After heating the flask to 100 °C to liquify the agar, the bacterial suspension was vortexed to ensure a uniform suspension. The plates were stained after washing to ensure that all bacteria had been collected. The bacteria were enumerated in a Petroff-Hausser counting chamber using a microscope equipped with darkfield optics. To show

that the bacteria did not lyse or aggregate during the procedure, a suspension of bacteria in PBS containing 0.05% SDS was heated for 20 min in a boiling water bath and compared to the number detected in the unheated suspension. No significant difference was observed.

Number of new clonal variants per area interval. The colonized area was mathematically divided into equal concentric intervals of 100 mm². The frequency of new clonal variants appearing within sequential intervals of area was then examined. To do this, the radial distance from the inoculation point to the inner point of the pie-shaped wedge was measured. From this radial distance, the area enclosed at the time of expression was calculated. Then, the number of sectors originating within each 100 mm² interval of area were summed and plotted as a function of the distance of the interval midpoint from the inoculation site.

Data analysis. The statistical analyses of the data were performed on an Apple Macintosh computer using Statview SE + Graphics software (Abacus Concepts, Berkeley, CA, USA).

Results

Fig. 1 depicts circular interfacial colonies stained *in situ* beneath the agar. The zonal pattern did not develop when the incubation time was less than 24 h (Fig. 1a). After 72 h (Fig. 1b), the colonization was composed of three distinct zones: an outer ring, an inner ring, and an inner circle. The outer ring possessed additional fine structure in which the outermost edge was dense and enclosed another less dense ring. The width of the outer ring varied from 0.5 to 2 mm. The inner ring was uniformly turbid, lacked substructure, and ranged in width between 5 and 7 mm. The inner circular zone had granular composition throughout with small granules at the outer edge and larger granules closer to the inoculation site. Pie-shaped wedges or sectors were seen within the colonized area. The inner points of the sectors usually occurred distal from the inoculation point, which suggested they were initiated after inoculation. These sectors were composed of daughter variants having properties different from the parent bacterial population. For example, among other differences, the colony shown in Fig. 1(b) contained a sector with a narrower outer ring zone than the parent growth.

Viable bacteria could be recovered from the agar-dish interface by stabbing through the agar with a loop and streaking the bacteria on the surface of fresh agar medium in the conventional manner. The bacteria recovered from both the outer ring and the inner ring only yielded colonies with the SC morphology. These colonies corroded the underlying agar in the shape of a rimless pit. Bacteria recovered from the outer edge of the inner circle yielded colonies with both NSC and N morphologies, but none with the SC morphology. In older colonizations, fewer viable bacteria were recovered from the area adjacent to the inoculation point, and those

Fig. 1. Circular colony formation at the interface of the agar and Petri dish bottom by *M. bovis* isolate MED72. Growth was stained with Coomassie brilliant blue R250. (a) Growth after 24 h at 35 °C, 5% CO₂. (b) Growth after 72 h. In the older growth three zones are visible: an outer ring (OR), an inner ring (IR) and an inner circle (IC). The inoculation site is indicated by I. Two sectors, each indicated by S, are visible in the 72 h colony. Note the difference in width of the outer ring zone for the most prominent of the sectors. Bars, 1 cm.

that were had only the N morphology. The NSC colonies corroded agar in the shape of an inverted 'fried egg' or a wide-rimmed bowl. The N colonies did not corrode the agar. When bacteria from these colonies were stabbed through the agar, only the corroding forms colonized the interface.

Bacteria adhering to the Petri dishes were stained with both Coomassie blue and Gram's stain after the agar was removed. Technical difficulties encountered in removal of the agar caused disruption of the adherence pattern. Even so, nearly all the bacteria in some zones remained on the dish surface while most of the bacteria in other zones separated with the agar. Typically, the inner ring bacteria stayed on the dish, while the bacteria in the outer ring and inner circle were removed with the agar. Consequently, the inner ring stained heavily, while the inner circle and outer ring stained lightly (Fig. 2b). Some sectors selectively adhered to the dish. Upon staining and microscopic examination, Gram-negative diplobacilli characteristic of *M. bovis* were observed.

When nitrocellulose blots of the underside of the agar were probed with the pilus-specific antisera, the antisera binding pattern complemented the pattern detected after Coomassie blue staining. The antisera were bound strongly by the outer ring and the inner circle, but weakly

by the inner ring. This was also seen for some of the sectors. When a sector bound the Coomassie stain on the dish, the anti-pilus sera did not bind at that position on the nitrocellulose blot. These features are illustrated in Fig. 2(c).

The twitching motility of *M. bovis* was observed using darkfield microscopy. Bacteria in both the outer rings twitched as described by Henrichsen *et al.* (1972). Individual adherent bacteria in these rings spontaneously moved short distances, while individual bacteria in the inner circle remained fixed in position. A summary of some characteristics of the different zones is given in Table 1.

To elucidate whether proliferation occurred only at the outer periphery, it was necessary to show that the bacterial density was constant regardless of the total area colonized. To do this, the number of bacteria associated with 22 different sized colonizations (50 to 3000 mm²) was determined. The Petroff-Hausser counting method was selected so that non-viable and aggregated bacteria would be included in the determination. A linear relationship between the number of bacteria and colonized area was observed. The linear regression equation for these data was

$$N_b = (4.23 \times 10^5 \text{ bacteria mm}^{-2})A_c + 7.28 \times 10^7 \quad (1)$$

Fig. 2. Examination of a colony by three methods. (a) Tracing of observable features of the colony prior to removal of the agar. (b) Bacteria adhering to the Petri dish stained with Coomassie blue. (c) Autoradiograph of a nitrocellulose blot of the agar removed from the dish and probed with rabbit anti-pilus sera. The orientation is the same for each panel; bars, 1 cm. The outer ring, inner ring and inner circle are as designated in Fig. 1. The sectors are numbered clockwise. Note that the sector designated S₆ was not seen until after the agar was removed from the dish.

In this equation, N_b is the number of bacteria in the colonized area, and A_c is the colonized area. The correlation coefficient was 0.96, the F value was 280, and the P value was less than 0.0001. This analysis indicated a linear relationship with the slope of the line being the

average density of the bacteria within the colonies. Based on this density and an average diplobacillus size of 0.5 by 1.75 μm (Breed *et al.*, 1957), the bacteria in this experiment were estimated to have occurred in a layer of about one bacterium thick. In another experiment, using

Fig. 3. Distribution of sector expression as a function of distance of the colonized area from the inoculation site. The number of sectors originating within each 100 mm² concentric area interval was plotted against the midpoint of the area interval. The data were compiled from an examination of 59 colonies and normalized to that of a single colony. An average of 6.0 (SD = 3.2) sectors occurred in each colony and the number per colony ranged from zero to eighteen.

Table 1. Summary of bacterial characteristics

Characteristic	Zone		
	Outer ring	Inner ring	Inner circle
Bacterial distribution within zone	Greatest at very outer edge	Uniform	Granular
Colonial form of re-cultured bacteria*	SC	SC	NSC or N
Adhesion of bacteria to Petri dish†	Weak	Strong	Weak
Binding of anti-pilus antibodies to blot†	Strong	Weak	Strong
Twitching motility	Yes	Yes	No

* SC, spreading-corroding; N, non-spreading, non-corroding; NSC, intermediate.

† In one experiment out of ten, these two characteristics were reversed. In this experiment, the bacteria in the outer ring and inner circle adhered to the dish and those in the inner ring did not. The adhesion of bacteria to the dish and the binding of antibodies to the blot of the agar were always complementary. This suggested that the most of the pili remained associated with the bacteria, so that when the bacteria of a particular zone bound to the nitrocellulose so did the pili. Similarly, if most of the bacteria of that zone stayed on the dish, the pili stayed too.

the FLA64(6) isolate, the thickness was estimated to be about fourteen layers. In both experiments, the density was nearly constant confirming that the bacterial population was only increasing at the outer edge of the colonies.

The distribution of observable sectors, as a function of colonized area, is illustrated in Fig. 3. The frequency of new sector expression was greatest in the early part of the colonization process, but asymptotically approached

zero as the colonization progressed. However, the number of sectors expressed within the first interval after inoculation was always lower than in the three subsequent intervals in all replicate experiments.

Because the bacterial density was constant, the total number of bacteria within the colonies was directly proportional to the area colonized. Thus, the colonization rate could be determined from plots of the colony diameter versus time. In the same experiment for which the sector frequency was examined, five colonizations were drawn at random from the 59 plates and analysed in this way. Although a regression analysis showed that these data fit a straight line equation very well, the line extrapolated to a negative diameter at zero time. This problem was especially evident when the growth of an individual colony was followed (Fig. 4). For this reason, a second-order polynomial equation was applied to the data as a way to characterize the growth. The data from the five plates gave the following equation:

$$D = 0.48 + 0.346t + 0.001t^2 \quad (2)$$

In this equation, D is the diameter in mm and t the time in h. The intercept at time-zero had a positive value of less than half a millimetre, while the straight line equation found using the same data set gave a negative intercept of 3 mm.

Reproducibility

The zonal ring pattern was highly reproducible, and every colony examined possessed three distinct zones. The experiment examining the frequency of sector variants as a function of distance from the inoculation site was performed three times with comparable results.

Fig. 4. Example of radial growth of a single *M. bovis* interfacial colony. The growth occurred in two phases. In the first phase, the diameter increased more slowly with time and was not linear. In the second phase, the diameter increased linearly and extrapolated to a starting diameter of -7.4 mm.

Discussion

M. bovis was able to colonize the interface between the agar and the Petri dish bottom. The resulting colonies were circular and continued their radial expansion for as long as medium remained available. The bacterial density in these colonies was nearly uniform and estimated to be from one to as many as fourteen bacterial cells thick. The uniformity of colonial density and circularity meant that the colonization progressed outward in the shape of a very thin flat cylinder or disk, fitting the model of growth proposed by Pirt (1967). When Pirt examined the radial rate of expansion of agar-grown colonies, he noted three growth phases. He did not characterize the growth phase occurring immediately after inoculation, but speculated that it might be exponential in nature. The second phase was one of linear radial expansion, and in the third phase, radial expansion slowed and then declined. Only two growth phases were seen for the interfacial *M. bovis* colonies (Fig. 4). These consisted of an initial slow, non-linear radial expansion phase followed by a second linear radial expansion phase. Pirt's third phase was apparently abrogated by the high rate of growth away from the original inoculation site into areas with higher nutrient concentrations. The problem with fitting all the data to a straight line matching the second phase growth was that these lines extrapolated to a negative value for the diameter of the colony at zero time. This suggested that the nature of the early colonial growth had to be different from that occurring after the linear expansion phase began. Non-linear expansion during the earliest phase has been reported for colonies of other species on agar

media (Pirt, 1967; Palumbo *et al.*, 1971; Jones & Gray, 1978; Wimpenny, 1979). Pirt suggested, based on observations by Plomley (1959) on growth of the fungus *Chaetomium* sp., that the initial phase in bacterial colony formation was likely to be exponential. The results of Jones & Gray (1978) and of Wimpenny (1979) confirmed this. In the present experiments, rather than describe the radial growth of the *M. bovis* using two separate equations, the data were fitted to a second-order polynomial equation (equation 2). This equation not only described the radial growth of the colonies, but could be mathematically differentiated to provide an equation describing the change in colonization rate as a function of time.

An important feature of Pirt's model of colony formation was that it required the replicative behaviour of the bacteria to be different at the edge from that in the interior of the colony. This requirement is necessary in his model since the colony is confined to being a thin flat cylinder of uniform height. Because of this, he proposed that the primary site of bacterial replication during linear growth was within a narrow ring at the periphery of the colony, and that the bacteria in the interior of the colony were non-growing. The results seen for the *M. bovis* colonization fit this model. However, the model did not explain the three distinct concentric growth zones. The existence of these different zones was likely due to differentiation by the bacteria into different phase variants with visually different properties. *M. bovis* has phase variants with distinctive colonial morphologies (Pedersen *et al.*, 1972), and variants with these different phenotypes were isolated from different zones. Thus, as the bacteria progressed from a proliferative to a non-growing form, they changed phase type. The intermediate zone, i.e. the inner ring, contained bacteria in the midst of transition and the width of this ring may be a measure of the time required for the transition to occur. The inner ring was typically between 5 and 7 mm wide, so during linear colony growth, the time needed for transition was between 23 and 32 h. It was also noted that the widths of the ring zones were uniform regardless of the direction of growth. Concentration gradients inevitably develop during colonization (Jones & Gray, 1978), and a change in concentration of either a nutrient, a metabolite, or possibly a signal associated with the shut-down of a metabolic pathway, might be the cue triggering differentiation. A change in pH seemed unlikely to be the cue since the same zonal pattern was seen whether the *M. bovis* was grown on GC medium base, a well-buffered medium, or on the weakly buffered Mueller-Hinton medium.

Only bacteria from colonies with either the SC or NSC morphology could colonize the interface. Both these forms were piliated, suggesting that pilus-associated

twitching motility might account for the rapid expansion of the interfacial colonies (Pedersen *et al.*, 1972). Furthermore, only the piliated SC form could be recovered from the outer ring, the site of active colonization. Additional evidence for this association was seen after colonies were blotted onto nitrocellulose and probed with anti-pilus antibodies. Pili were detected in the outer ring and in the inner circle zones, but only weakly in the inner ring zone. Their presence in the inner circle zone did not seem consistent with the recovery of only the non-piliated N form near the inoculation site. The pili detected there may have been shed by piliated forms prior to their transition to N forms. The different amounts of pili detected in each zone were inversely correlated with the pattern of bacterial adherence to the dish (Fig. 2*b*). This complementary pattern meant that the bacteria and pili tended to separate together when the agar was removed from the dish. So when most of the bacteria separated with the agar, most of the pili separated with them as a part of the same matrix.

The recovery of different colonial or phase types from the zones indicates that *M. bovis* has a special mechanism to deal with down-shifts in replication. The association of phase variants with separate proliferative and quiescent forms may also occur for other bacterial species (Siegele & Kolter, 1992). Lewis & Gattie (1990, 1991) have proposed that multiple proliferative forms provide a mechanism for the survival of bacteria that inhabit soil, fresh water lakes and seas. In the case of pathogenic bacteria, Gottschal (1990) has suggested that the lower metabolic demands of a dormant form might allow bacteria to survive in some host environments better than an actively replicating form. If this is the case for *M. bovis*, it may be a mechanism for surviving some portion of its life cycle. For example, the time spent outside its host during transmission or in some phase of eluding the host's immune system.

Clonal variants had a variety of phenotypes and a very different origin than the phase variants. Their sector shape suggested a heritable nature. The appearance of new clonal variants was not random. Excluding the interval nearest the inoculation site, after the colonies were mathematically divided into concentric rings of equal area, more new daughter variants were observed within the intervals close to the inoculation site than in the more remote intervals. This implied that the inoculation step somehow affected the early stages of the colonization process and contributed to the post-inoculation florescence or burst of new daughter variants. Radial expansion of the interfacial colonies was slowest in the earliest stages of the colonization. Thus, there seemed a connection between the frequency of clonal expression and the time the parent bacteria needed to colonize virgin area. The strongest evidence

Fig. 5. Correlation of number of sectors expressed per area interval with the inverse of the rate of colonization of area. The rate of colonization (dA/dt) was determined for the midpoint of each interval using equation 2. The number of sectors achieving expression in each interval (N_i) was taken from the data shown in Fig. 3. The linear regression equation for this line is $N_i = 7.2/(dA/dt) - 0.19$. The correlation coefficient for this calculation was 0.96, the F distribution value was 326, and the P value less than 0.0001. 'Point A', representing the datum point for the first interval after the inoculation, was excluded from the regression analysis because of its obvious divergence. Possible reasons for the divergence of this point are discussed in the text.

for this was seen in a regression analysis (Fig. 5) showing a linear relationship between the number of new variants per interval and the inverse of the rate of expansion in area calculated from equation 2. This relationship implied there was competition between the parent and the daughter variants, and only the daughter variants that became competent within a brief allotted time survived.

The kinetics of daughter variant expression bears a similarity to the phenotypic lag time required by antibiotic-resistant mutants to become competent, and are likely to be different for each variant type (Luria & Delbrück, 1943). The lag in daughter variant expression may be one reason the greatest number of clonal variants appeared in the second and third interval areas of the experiment, while relatively few were expressed in the first interval. Another reason for the paucity of variant expression in the first interval may be due to an adaptive growth lag subsequent to inoculation into fresh media. An adaptive lag would be expected to be less stressful on the parent variant than a nascent daughter variant. A combination of both factors may explain the lull in variant expression immediately after inoculation and just prior to the burst observed within *M. bovis* as well as *E. coli* colonies (Shapiro, 1984; Hall, 1988).

Finally, the relationship between the parental colonization rate and the kinetics of daughter variant expression may modulate not only the frequency of daughter variant expression but also the kind of variant. The competition between the daughter and parent variants provides a way of sorting the kinds of daughter variants expressed. The bacterium could take advantage of this by arranging its genome so that variant types beneficial to survival have a faster track for achieving competence than other variant types. This may explain the high frequency of switching between pilin serotypes observed for *M. bovis* (Marrs *et al.*, 1985). If this concept is correct, then the parent variant has a degree of control over the expression of what kind as well as the frequency of new clonal variants.

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