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Interactions among Millipedes (Diplopoda) and their Intestinal Bacteria

by

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Abstract: (1) 142 *Pseudomonas* strains isolated from the faeces of *Glomeris hexasticha* BRANDT (Diplopoda) and *Tomocerus longicornis* (MÜLLER) (Collembola) were compared and identified as members of the same variety of *Pseudomonas stutzeri*. (2) The utilization abilities of 269 faecal bacterial and actinomycete strains of millipedes and woodlice were studied in relation to 47 organic compounds. The results are discussed. (3) The antagonistic interactions between indigenous gut nocardioforms and bacteria of *Glomeris hexasticha* were demonstrated. (4). The absolute predominance of non fermenting, non *Oerskovia*-like nocardioform actinomycetes has been shown in the faecal microbiota of the Indian millipede *Trigonoiulus lumbricinus* (GERSTÄCKER).

1. Introduction:

Complex microbial communities composed of many species of partly true soil-bacteria (*Bacillus, Micrococcus, Clostridium, Corynebacterium,* etc.) and partly typical intestinal ones (*Klebsiella, Enterobacter,* etc.; BALEUX & VIVARES 1974, SZABO et al. 1983, CONTRERAS 1990) as well as actinomycetes (DZINGOV et al. 1982, SZABO et al. 1983, JAGER et al. 1983, CHU et al. 1987) and fungi (TAYLOR 1982) colonize the mid- and hindgut contents of millipedes. In the microhabitats of the latter these microorganisms (ANDERSON & BIGNELL 1980, HANLON 1981, ANDERSON et al. 1983), their extracellular enzymes (KHEIRALLAH 1979, TAYLOR 1982, SZABO et al. 1990) and the host animals' own enzymes (NUNEZ & CRAWFORD 1976, NEUHAUSER & HARTENSTEIN 1978, BECK & FRIEBE 1981) can interact very intricately. Millipedes can select in their gut and cultivate very rare soil microbes (SZABO et al. 1985), which will quickly disappear again from their deposited faecal pellets (MARIALIGETI et al. 1985, HEYDRICH & SZABO 1990). At present only few data are available on the species composition, biochemical abilities and antagonistic-cooperative interactions of the members of millipedes' gut microbiotas in relation to the host animal metabolism. Below we present some selected results of our latest studies on the gut communities of these animals.

2. Materials and Methods:

Figure 1 demonstrates that series of methods which we use for studying the millipede gut microbiotas: adult specimens of the very same species are collected aseptically in their feeding habitats (1) and their body surfaces are liberated with sterile brush from the adhering soil- and plant particles (2). Then the animals are placed on the surface of sterile hard synthetic agar plates (3) on which they move around and the microbes adhering to their legs remain on or incorporate in the agar where they will be able to develop into colonies after 3 - 10 days incubation.

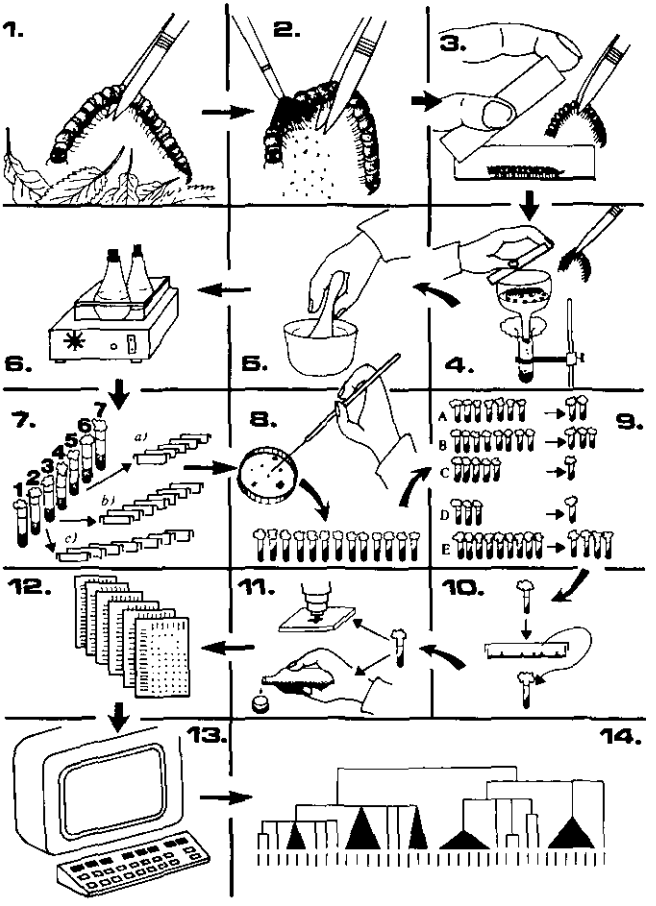

Fig. 1: A simplified sketch for demonstrating the order of methods used to study the gut microbiotas of millipedes and other soil invertebrates.

These animals will be then transferred onto a sterile grid over a sterile glass vessel (4) to collect their freshly laid faecal pellets. The latter will be mechanically disintegrated under sterile circumstances (5), suspended in sterile tap water, further homogenized on a shaker (6), serially diluted (7) and plated onto agar media of at least three different (7: a - c) chemical compositions. After incubation at 28° C in the dark the developed individual colonies are picked up randomly and transferred to agar slants of the same composition (8). In this way, a large number of isolates, frequently over 1000, are obtained. These can be grouped tentatively into a series of separate assemblages of similar organisms (e.g. 9. A - E), according to their easily identifiable physiological and cultural-morphological properties. After this from every individual group of similar isolates a number of representative strains will be selected in accordance with the total number of isolates placed in the group in question, for further detailed studies. These strains are purified by reisolations (10), studied light- and electromicroscopically, submitted to a series of at least 150 physiological and biochemical tests and also analysed for the chemical composition of their whole cells or their cell-wall preparations (11). The list of diagnostic properties used for the standard descriptions of strains and the methods employed for detecting them are given in our earlier publications (SZABO 1974, CHU et al. 1987, SZABO et al. 1990). The obtained data are then coded for computer aided numerical analyses (12) and all of the examined strains among them as well as the authentic reference strains are compared to show the extent of their

similarities. Between every pair of strains a similarity index is calculated (13) and finally a dendrogram is constructed (14) which demonstrates the phenetic similarities (in per cent) or taxonomic relationships among all of the compared strains and their groups.

3. Results and Discussion:

3.1. The Distribution of individual Species of Bacteria in Gut Habitats of different Invertebrate Animals:

Bacterial species migrate permanently among plant, animal and soil microenvironments. Through many habitats they can travel only passively as inactive propagules, but in many others they can become established and also reproduce. Under adequate environmental conditions they can attain large population densities as locally predominant organisms. Knowledge on these pathways of migration is of great importance if we are to understand complex soil biological events. For example, *Pseudomonas fluorescens* occurs in the soil matrix chiefly only as solitary cells but it can form dense epiphytic populations on developing root hairs of *Robinia pseudoacacia.* After the decay of the roots, however, its cells disseminate again in the soil. If a few of these cells can pass with the consumed plant and soil matter through the digestive canal of the larvae of April-fly (*Bibio marci*) they will multiply in the latter's hind-gut and become predominant gut colonizers (SZABO 1974). *Oerskovia turbata,* a well defined (BERGEY's Manual Vol. 4) and widely distributed nocardioform actinomycete species, can form intestinal populations in adult and juvenile individuals of both Diplopoda, Isopoda and Oligochaeta spp. in different soil types all over the world (SZABO et al. 1990). According to our latest findings another species of *Pseudomonas, P. stutzeri* may be problably also characterized by a multispecies host animal range. A variety of *P. stutzeri* proved to be able to colonize successfully the gut environments of a millipede (*Glomeris hexasticha*) as well as a springtail (*Tomocerus longicornis*) attaining a prominent community position in both animals.

The data in Table 1 shows a comparison of certain selected diagnostic features of two series of *P. stutzeri* strains. Only the nitrate reduction test showed a consistently detectable difference between them: in contrast to the *P. stutzeri* strains of *G. hexasticha,* all of the 91 strains isolated from a composite fresh faecal sample of 130 specimens of *T. longicornis,* proved to be able to reduce nitrates. Besides, they showed generally weak but positive oxydase reaction, while the pseudomonads of *G. hexasticha* decarboxylated amino acids with relatively weak intensities. The taxonomic identity at species level of the members of the two populations seems to be obvious.

3.2. Differences in Physiological Abilities of Millipede Gut Microbiotas:

There are considerable differences both in the actual physiological activities and in the potential biochemical capabilities of the gut microbiotas of invertebrate animals. The former is, in general, studied by measuring the enzymatic, CO_2-production, and other activities of gut contents containing the total microbiota or its lysates. The latter may be clarified by studying the biochemical activity spectra of the individual isolated members (strains) of gut communities, and in this respect our knowledge is much more meagre. Table 2 shows selected data on the utilization abilities of 269 representative gut strains. The listed carbon compounds were added to the medium as sole sources of C combined with $(NH_4)_2SO_4$ as the nitrogen source. Nitrogen-containing compounds (e.g. chitin) were also added as sole C- and N-sources. Benzoate was utilized only by 13 strains isolated mostly from the faeces of a Spirostreptidae sp. and an *Amphelictogon* sp. (Chelodesmidae) collected in sugar-cane plantations in Cuba. On the other hand, many strains can grow with citrate, acetate, aesculin, lactose and especially with glycerol. No oxalate utilizer was found among the 115 *Glomeris*-strains although oxalate positive strains occurred in low numbers in all other compared gut microbiotas. Salicilate utilizers were detected only among the Cuban Diplopoda-faecal isolates, and interestingly the number of cellulose decomposers among the aerobic and facultatively an-

Table 1: A comparison of two intestinal populations of *Pseudomonas stutzeri* *).

Host animal	*Glomeris hexasticha*	*Tomocerus longicornis*
No. of studied strains	51	91
Gram staining	negative	negative
Resting stages	no	no
Colour of colonies	cream to bright orange	
Surface of colonies	strongly wrinkled	
Motile by means of	a single polar flagellum	
Metabolism	oxidative	oxidative
Oxydase	+ (51)	± (91)
Catalase	+ (51)	+ (91)
Urease	+ (51)	+ (91)
Phosphatase	+ (51)	+ (91)
Indole	+ (51)	var (46)
Methyl-red	− (51)	− (91)
Voges-Proskauer	− (51)	− (91)
Nitrate reduction	− (51)	+ (91)
Growth at 40 °C	− (51)	− (91)
Hydrolysis of		
starch	+ (51)	+ (91)
gelatin	+ (51)	+ (87)
Tween−80	+ (51)	+ (91)
aesculin	+ (51)	+ (91)
hypoxanthine	+ (51)	+ (91)
Decarboxylases		
ornithine	± (51)	var. (40)
arginine	± (51)	var. (65)
lysine	± (51)	var. (38)
Utilization of		
arabinose	+ (51)	+ (91)
xylose	+ (51)	+ (91)
rhamnose	+ (51)	+ (91)

*) var: variable results; in parentheses the numbers of positive strains are given.

aerobic gut bacteria of Diplopoda is only low, as in the cases of Spirobolidae spp. (collected in *Eucalyptus* forest litter in Ecuador) or even zero. Strains which can decompose partially hydrolysed chitin have always been isolated in low number. It is not probable, that in the gut of millipedes microbial decomposition of native chitin takes place. Striking differences in physiological abilities of strains isolated from the faeces of woodlice or millipedes were not detected. Gut microbiotas frequently show differences not only at species- but also at individual levels of the host animal.

Table 2: Decomposition and utilization of different chemical compounds by selected representative strains of aerobic and facultatively anaerobic bacteria and actinomycetes isolated from the gut contents and faecal pellets of some invertebrate animals. (The numbers of positive strains are presented; numbers in parentheses: not all strains were tested; ND: no data.)

Host animal	*Glomeris hexasticha* (Diplopoda)	*Mesoniscus graniger* (Isopoda)	Spirobolidae spp. (Diplopoda)	Spirostreptidae sp. and *Amphelictogon* sp. (Diplopoda)
No. of studied strains	115	65	41	48
acetate	28	53	18	42
adenine	82	ND	5	ND
adonitol	ND	1	3	20
aesculin	111	56	13	29
arbutin	112	ND	22	ND
benzoate	–	–	1	12
cellulose	–	–	3	–
chitin	ND	3	–	10
citrate	35	22	15	35
dextrin	ND	17	24	(15)
DNA	92	*18*	*ND*	*(6)*
dulcitol	15	–	1	21
elastin	–	ND	–	ND
gelatin	80	ND	37	28
glycerol	109	35	18	44
guanine	–	ND	–	ND
hypoxanthine	64	23	8	22
inositol	20	1	3	21
inulin	ND	16	9	ND
lactose	92	14	13	33
lecithin	ND	33	ND	ND
maltose	*107*	*ND*	*30*	*45*
mannitol	44	8	9	35
melizitose	ND	3	6	19
oxalate	–	9	2	11
raffinose	15	2	7	13
rhamnose	93	8	ND	(–)
ribose	ND	22	ND	ND
RNA	106	13	ND	(–)
salicylate	–	–	–	6
salicin	61	32	15	41
sorbitol	ND	–	2	ND
starch	*71*	*22*	*21*	33
sucrose	ND	–	ND	(21)
testosterone	16	ND	–	ND
tributyrin	115	64	34	48
Tween-40	114	52	26	37
Tween-80	108	3	ND	34
urea	108	26	12	26
xanthine	5	19	–	–
xylitol	ND	1	ND	ND
xylose	98	17	13	33

3.3. Antagonistic Interrelationships among Gut Microbes of Millipedes:

In the millipede intestinal microcosm there exists a continuously changing equilibrium among the interacting populations of the indigenous bacterial and actinomycete species. A particular type of nocardioform actinomycetes possessing a diamino-butyric acid (DAB) containing cell wall

proved to be an indigenous member of the gut biota of *Glomeris hexasticha* (CHU et al. 1987). Gut isolates (strains Nos 9/858, 983, etc.) of this nocardioform are physiologically relatively inactive organisms, do not produce antibiotic substances and can grow relatively slowly. However, it can be a frequent member of the gut community. We studied the coexistence of six such nocardioform strains with 11 selected representative strains of other common or codominant species of intestinal bacteria (*Pseudomonas*, Enterobacteriaceae spp.) of *G. hexasticha*. Strains were cocultivated by pairs in 100 ml nutrient broths in Erlenmayer flasks of 250 ml volume. Broths were simultaneously coinoculated with approximately equal cell-numbers of the contrasted partners using their suspensions. Incubation lasted 20 days at 28° C. A gentle periodical shaking was employed. Each combination of partners was studied in three parallel broth cultures. At the end of incubation the counting of the cell densities of the individual partners was carried out by the plate-count technique and reisolations. The incubated plates were exposed to natural light before counting because these nocardioforms are photochromogenic organisms showing characteristic yellow colourization following such exposure. The nocadioform/bacteria ratio was calculated.

The results are demonstrated in Table 3. As can be seen some bacterial strains (Nos 8/874, 9/904, 1001, 5/971) could completely eliminate all of the nocardioform partners. Others (1030, 6/627, 9/890) showed such very strong antagonistic potency only against certain strains of nocardioforms. On the other hand, there were also very sensitive gut bacterial strains (940, 3/267), which proved to be unable to survive at all in the presence of nocardioforms. Finally, strains 9/734 and 4/226 showed a tendency to coexist with nocardioforms. Although all of these gut nocardioform strains can be considered as members of a single species (see CHU et al. 1987), their interactive characteristics are very different. Perhaps this fact lies behind the detected coexistence of nocardioforms with bacteria in the gut of *G. hexasticha*.

Table 3: Mutual antagonistic interrelationships in nutrient broth among intestinal nocardioform strains (DAB in the cell-wall) and different bacterial strains isolated from the faeces of *Glomeris hexasticha*. (In the boxes the nocardioform: bacteria ratio is given after 20 days incubation, B: on the 20th day only the bacterial strain was living; N: only the nocardioform strain survived the cocultivation.)

		Strains of intestinal bacteria										
		9/734	940	1030	6/627	8/874	9/904	1001	4/226	5/971	9/890	3/267
Intestinal nocardioform strains	9/858	16 : 1	N	1 : 21	B	B	B	B	7 : 1	B	B	N
	983	5 : 1	N	B	B	B	B	B	67 : 1	B	1 : 8	N
	6/620	128 : 1	N	1 : 63	1 : 13	B	B	B	75 : 1	B	1 : 1	N
	6/408	N	N	B	B	B	B	B	N	B	B	N
	181	1,5 : 1	N	B	B	B	B	B	N	B	B	N
	1017	57 : 1	N	B	B	B	B	B	N	B	B	N

3.4. Non Fermenting Nocardioforms in the Gut of Millipedes:

Facultatively anaerobic, glucose fermenting *Oerskovia*-type nocardioform actinomycetes which can develop disintegrating yellow substrate mycelium and incorporate lysine as stable constituent in their cell wall (cell-wall-type No VI) are frequent indigenous members of millipede gut microbiotas, but occur in soils only sporadically (SZABO 1990). *Glomeris hexasticha* was the first millipede in the faeces of which taxonomically hardly identifiable, non fermenting nocardioforms were detected in realtively large number (CHU et al. 1987). These *Glomeris* isolates different from Oerskovia both physiologically and in the chemical composition of their cell wall. It is remarkable that non fermenting nocardioforms occur in low number in the gut contents of many other millipedes too, but that these are partly defective oerskovias.

During the last two years we studied the composition of faecal microbiotas of specimens of *Trigonoiulus lumbricinus* (GERSTÄCKER) (Pachybolidae) collected near to Bangalore (India). Although already we have information on the occurrence of nocardioforms in the gut of tropical millipedes (RAKHMO & SZABO 1990, HEYDRICH & SZABO 1990), their absolute predominace in the *Trigonoiulus* faecal samples was still surprising. Besides, none of the isolated nocardioform strains proved to be facultatively fermentative. Fig. 2 shows a dendrogram presenting the similarities of 75 representative *Trigonoiulus* faecal strains. The SOKAL-MICHENER-coefficient (SZABO 1974) was calculated on the basis of 162 coded features by an IBM PC AT-type computer. Cluster analysis (single linkage) and the construction of the dendrogram were based on 12150 data. The computer created altogether 9 groups of similar strains, groups 4, 5 and 8 were further subgrouped. Taxonomically the former represent species levels, the latter variety levels. Among the members of these groups only those of group 9 proved to be true fermenters and were identified as *Enterobacter* sp. Strains of groups 1, 2, 6 and 7 represent gut bacterial fractions of low population densities. Among these the strains of groups 6 and 7 showed diagnostic properties of *Micrococcus*, a genus which otherwise frequently occurs in invertebrate gut milieus. The large group 8 which has been differentiated into five (a - e) subgroups comprises coryneform bacteria and represents the second most dense fraction of the *Trigonoiulus* gut biota. Finally, groups 3, 4 and 5 as well as their subgroups involve strains of the absolutely predominant gut fraction and represent many varieties of at least three separate species of non fermenting, non *Oerskovia*-type nocardioform actinomycetes. We are attempting to identify these taxonomically less well known microorganisms.

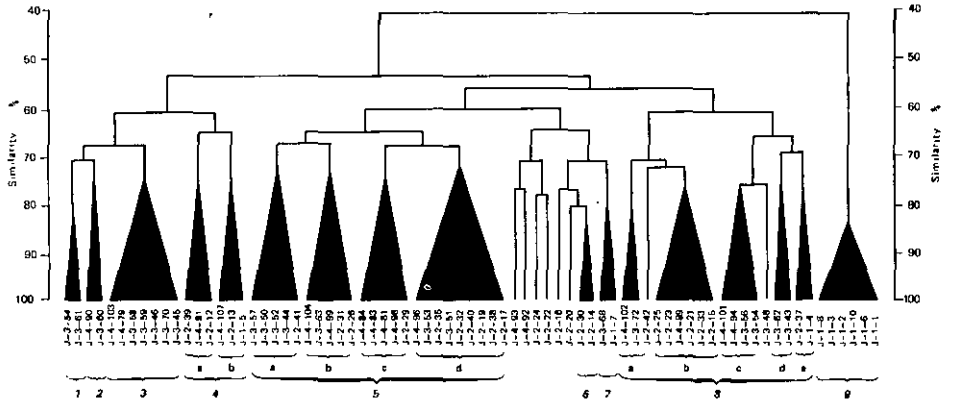

Fig. 2: Dendrogram showing the phenetic relationships in percent among 75 selected representative actinomycete (non fermenting nocardioform spp. and their varieties: Groups 3., 4.a-b and 5.a-d) and bacterial (Micrococcus spp.: Groups 6. and 7.; five varieties of a coryneform sp.: Group 8.a-e); Enterobacter sp.: Group 9.; etc) strains isolated from the faeces of the Indian millipede *Trigonoiulus lumbricinus*.

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