Male-Killing Bacteria in a Parasitic Wasp

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A rod-shaped bacterium has been isolated that kills male eggs of the wasp *Nasonia vitripennis*, a pupal parasite of flies. Only some wasps of this species express this son-killer trait, and these wasps have bacterial infections in various organs. The bacterium was isolated from son-killer wasp tissue and from the hemolymph of fly pupae parasitized by wasps expressing the son-killer trait. Bacteria are apparently transferred to parasitized fly pupae during wasp oviposition, and developing wasp offspring are subsequently infected perorally. Sex-ratio distortion by microorganisms is found in a variety of plants and animals. The infectious peroral transmission of this trait is in contrast to the typical pattern of cytoplasmic inheritance of sex-ratio distortion in these other systems.

A VARIETY OF EUKARYOTIC SPECIES harbor maternally transmitted microorganisms that cause female-biased sex ratios. The sex ratio is typically biased by the mortality of male offspring or by alteration of sex determination (*1–6*). Diverse microbial taxa have been identified as distorters of the sex ratio. For example, cytoplasmically transmitted spiroplasmas kill males in several species of *Drosophila* (*2*), and cytoplasmically transmitted microsporidia kill males in several mosquito species. Microsporidia cause sex reversal of males to functional females in certain amphipod species (*3*). *Rickettsia tsutsugamushi*, the causative agent of scrub typhus, has been implicated as a cause of all-female progeny in its mite host (*4*). Viruses have been implicated in certain cases of male sterility in plants (*1, 5*). The widespread occurrence of sex-ratio microorganisms in diverse taxa raises the question of why these organisms have been selectively favored in evolution. Various investigators have speculated on this topic, but the selective advantage of sex-ratio distortion to either the microorganism or its host has not been thoroughly investigated in any species.

The most extensive assemblage of sex-ratio distorters so far discovered occurs in the gregarious wasp *Nasonia vitripennis* (Hymenoptera, Pteromalidae), which parasitizes the pupae of various fly species (*6–8*). As in other Hymenoptera, the wasp has haplodiploid sex determination: unfertilized (haploid) eggs develop into males, and fertilized (diploid) eggs develop into females. This mode of sex determination gives *N. vitripennis* control over the sex ratio of the offspring, and individual wasps make from 0 to 90 percent daughters depending upon circumstances (*9*). Thus, sex-ratio variability is attributable both to facultative shifts by "normal" wasps and to the presence of extrachromosomal sex-ratio distorters in natural populations. Recent studies have disclosed three extrachromosomal factors: (i) paternal sex ratio (*psr*), paternally inherited and causing all-male families (*10*); (ii) maternal sex ratio (*msr*), maternally inherited and causing 97 percent female families; and (iii) son-killer (*sk*), maternally and contagiously transmitted and inducing the deaths of male eggs in host pupae. About 40 percent of female wasps in the field carry one of these sex-ratio distorters (*7*).

The etiology of *psr* and *msr* is unknown. Yet histopathological studies on *sk*-strain specimens have revealed that systemic and chronic bacterial infection is intimately associated with this trait (*8*). The *sk* trait is different from other sex-ratio distorters in that it can be infectiously transmitted among developing offspring within a host. This occurs when an *sk* female and an uninfected female parasitize the same host (*7*). Histopathological studies indicate that the bacteria are probably transferred to the fly host during parasitization and that they are then ingested by the developing wasp offspring; this results in the peroral transmission of the bacteria to the next generation (*8*). On the basis of these observations, we attempted to isolate the bacterium.

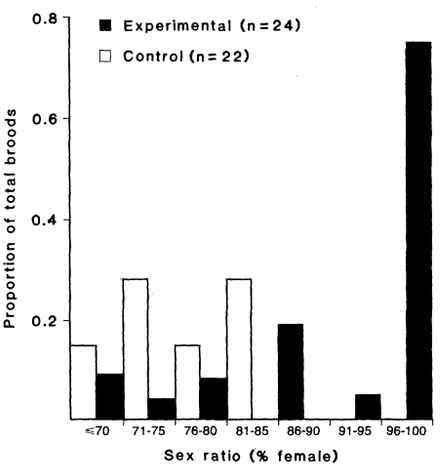

Fig. 1. The distribution of sex ratios produced by females that had developed in hosts injected with the slow-growth bacterium (experimental) versus hosts injected with broth only (control). Females from the bacterium-injected hosts show sex ratios characteristic of *sk* females.

This report describes the in vitro isolation of a rod-shaped bacterium from *sk*-wasp tissue and from the hemolymph of fly pupae parasitized by *sk* females. Experimental evidence indicates that this bacterium is the causative agent of the *sk* trait. These results represent the first in vitro culturing of a sex-ratio microorganism.

Wasp tissue was isolated by the following procedure. The *sk* female and control ("Lab 2" wild-type stock) female pupae were surface-sterilized first with 50 percent Chlorox, then with 5 percent mercuric chloride, and then with 70 percent ethanol, and were rinsed with sterile distilled water after treatment with each solution; samples were then cut into two to three parts and placed in broth tubes of either tryptic soy broth (TSB), brain-heart infusion (BHI), or thioglycolate broth (TB). The resultant growth was identified by streaking broth onto TSB agar (TSA), and isolates were then characterized by standard microbiological tests (*11*). For host hemolymph isolations, hosts parasitized by either virgin *sk* or control females mated to irradiated males were used (*12*). Since virgin *sk* females lay only haploid (male) eggs, there is very low egg hatch in wasps carrying the *sk* trait. Therefore, control females were mated to irradiated males since this also results in low egg hatch (*12*). Ninety-six hours after parasitization, the puparial exoskeleton of the fly host was removed, hosts were surface-sterilized with 50 percent Chlorox and then 70 percent ethanol, and the hemolymph was extracted with a syringe. Broth tubes were inoculated with approximately 0.05 ml of hemolymph, and resultant growth was identified by standard microbiological procedures.

No growth was observed in the TB media. However, after 5 days evidence of a slow-growth bacterium was observed in the majority of TSB and BHI tubes from *sk*-female (pupae) and *sk*-parasitized hosts but not from the control. Monocultures of a bacterium were found upon streaking broth onto plates. A few tubes from both groups were also contaminated with *Proteus vulgaris*, a common bacterial species associated with flesh flies. Data are presented in Table 1. Clearly, the slow-growth bacterium is associated with the *sk* strain but not with the

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Table 1. Results of bacterial isolations from *sk* and control (wild type) wasp pupae and hosts stung by either *sk* or control females. Results show that the slow-growth bacterium is associated with the *sk* trait.

System	Number of tubes with		
	No bacterial growth	Slow-growth bacterium	Contamination
sk Pupae	12	38	5
Control pupae	37	0	3
sk-Stung host	0	20	4
Control stung host	25	0	3

control strain. Twenty-six bacterial isolates were characterized by a set of 17 growth and biochemical features routinely used in microbiological testing (*11*). All the slow-growth isolates showed the same characteristic profile, suggesting that they are apparently the same bacterium. This bacterium has unusual characteristics (*13*) that have so far prevented its assignment to a taxonomic family. Collaborative efforts with the American Type Culture Collection are under way to further characterize the organism, which is likely to represent a new species.

The slow-growth bacterium was shown to be the causative agent of the *sk* trait on the basis of the following experiments. The slow-growth strain was maintained on TSA for 2 months. Control wasps (uninfected with the *sk* trait) were allowed to parasitize hosts. These hosts were injected 24 hours after parasitization with 0.44 μl of either (i) sterile TSB, (ii) slow-growth bacterial suspension (5.8×10^3 cells per injection) in TSB, or (iii) *P. vulgaris* suspension (isolated from an *sk*-stung host, 2.1×10^4 cells per injection) in TSB. Eleven days later, pupae were collected from the hosts and the resulting female adults were tested for the *sk* trait. These tests were conducted at two separate laboratories, and the results are shown in Table 2. In assaying for the *sk* trait, we allowed virgin females to parasitize hosts and then scored them for the presence of unhatched eggs 72 to 96 hours later (*7*). The results were unequivocal; 100 percent of scoreable wasps from hosts injected with the slow-growth bacterium showed the *sk* trait versus 0 percent for either of the controls. Females from the hosts injected with the presumptive *sk* bacterium showed both sex-ratio changes and a high proportion of dead male eggs. Seventy-five percent of the experimental females tested produced a sex ratio of 96 percent females or higher (Fig. 1). The highest sex ratio produced by a control was 90 percent. The two distributions are significantly different (Kruskall-Wallis test, $H = 15.84$, $df = 1$, $P < 0.001$). We are now using this procedure of infecting strains with the *sk* trait with consistent results.

Histopathological examination of male and female wasps developing from hosts injected with the slow-growth bacterium showed the typical pattern of bacterial tissue infections observed in the original *sk* strains (*8*). Despite harboring bacterial infections, males developing in injected hosts did not die and were otherwise apparently healthy, although this was not quantified. However, females from such hosts subsequently showed the high proportion of dead male eggs among their progeny characteristic of wasps with the *sk* trait. The bacterium was reisolated from both female tissue and from hosts parasitized by females from injected hosts but not from controls. Thus the bacterium is proven causative agent of the *sk* trait, in fulfillment of Koch's postulates (*14*).

Although 100 percent of females in the first generation showed the *sk* trait, the rate of transmission of this trait in successive generations was less than the rate observed in natural infections (*7*). These differences may be due to changes in the bacterium resulting from in vitro culturing.

Sex-ratio distortion by microbes occurs in a variety of host and microbial taxa. The *sk* trait is unique because of its peroral route of transmission to the next generation, in contrast to the cytoplasmic mode of transmission typical of other sex-ratio microorganisms. Transmission to other maternal lineages can occur when more than one female parasitizes the same host. The selective advantage to microbes that cause male lethality is not fully understood (*1*). Male lethality may indirectly increase the incidence of *sk* females in field populations since the resulting decrease in food competition within the host gives rise to large *sk* females. Large females in this wasp are more fecund and therefore would transmit the bacteria to more daughters. In contrast, male lethality is inconsequential to transmission of the bacteria so long as there are sufficient males remaining in the population to inseminate *sk* females.

Data suggest that the bacterium kills the male eggs by preventing the development of unfertilized eggs, which could otherwise develop parthenogenically in haplodiploid organisms (*15*). Therefore, the *sk* trait could possibly be used to elucidate the mechanisms for haploid parthenogenesis and sexual differentiation. This perorally transmitted bacterium may also prove useful in the biological control of pest insects.

Table 2. Results from experiments in which hosts were injected with either slow-growth bacterium, sterile broth, or *Proteus vulgaris*. Offspring from these hosts were then tested by scoring for egg lethality of virgins. Data show unequivocally that the slow-growth bacterium causes the *sk* trait. This trait was transmitted to subsequent generations, but at a lower rate than from natural infections. The progeny from individuals that lost the trait in generation 2 did not express *sk* in generation 3, consistent with results from natural infections.

Group	Number of hosts injected	Number of females		
		sk	Non-*sk*	Unknown
	Generation 1			
Slow-growth bacterium + broth	15	70	0	1
Broth only	14	0	72	0
P. vulgaris + broth	3	0	20	0
	Generation 2			
Slow-growth F_1 (*sk* parent)	0	45	31	3
Control F_1	0	0	71	1
	Generation 3			
Slow-growth F_2 (*sk* parent)	0	19	35	8
Slow-growth F_2 (uninfected parent)	0	0	14	2
Control parent	0	0	29	0

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10. The *psr* trait was first referred to as "daughterless" (Dl) (*6*). However, this identification caused confusion and is now changed to *psr* to reflect its paternal inheritance pattern.
11. The following tests were used to characterize isolates (results for the slow-growth bacterium, which causes the *sk* trait, are indicated in parentheses): (i) sugar fermentative tests: glucose (+), fructose (−), xylose (−), lactose (−), maltose (−), mannose (−), 10 percent lactose (−); (ii) miscellaneous tests: NO_2/N_2 (−); motility (−/+), catalase (+), oxidase (−), Gram stain (negative pleiomorphic rod); (iii) media growth tests (1,5 days, 25°C): TSB (−,+), BHI (−,+), thioglycolate (−,−), TSA (−,+). In addition, the *Proteus* isolates were identified by the Enteric Tec test kit.
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13. The bacterium shows an antibiotic sensitivity characteristic of Gram-negative organisms (polymyxin-, penicillin-, methicillin-, and erythromycin-resistant; gentamicin-, chloramphenicol-, tetracycline-, and sulfathiazole-sensitive). A fatty acid profile determined by M. Sasser (University of Delaware) did not match any in his current library (104 species) but was typologically most similar to that of Enterobacteraceae. R. Gherna (American Type Culture Collection) is further characterizing this species.
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16. J.H.W. thanks S. R. Simms and D. Ohasi for assistance in microbiological identification, D. Roberts and I. Schneider for use of facilities, and H. Schneider and I. Schneider for advice and guidance. S.W.S. thanks E. Patou for use of facilities, J. Phillips for discussions, and J. James for assistance. S.W.S. was supported by NSF grant DE-1381-19206 and NIH grant 5 T32 6MO7131. Helpful comments were provided by R. Gherna, K. Thorpe, B. Martin, J. Phillips, M. Raupp, and C. Reichelderfer.

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Three-Year Incidence of AIDS in Five Cohorts of HTLV-III–Infected Risk Group Members

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The incidence of the acquired immune deficiency syndrome (AIDS) among persons infected with human T-lymphotropic virus type III (HTLV-III) was evaluated prospectively among 725 persons who were at high risk of AIDS and had enrolled before October 1982 in cohort studies of homosexual men, parenteral drug users, and hemophiliacs. A total of 276 (38.1 percent) of the subjects were either HTLV-III seropositive at enrollment or developed HTLV-III antibodies subsequently. AIDS had developed in 28 (10.1 percent) of the seropositive subjects before August 1985. By actuarial survival calculations, the 3-year incidence of AIDS among all HTLV-III seropositive subjects was 34.2 percent in the cohort of homosexual men in Manhattan, New York, and 14.9 percent (range 8.0 to 17.2 percent) in the four other cohorts. Out of 117 subjects followed for a mean of 31 months after documented seroconversion, five (all hemophiliacs) developed AIDS 28 to 62 months after the estimated date of seroconversion, supporting the hypothesis that there is a long latency between acquisition of viral infection and the development of clinical AIDS. This long latency could account for the significantly higher AIDS incidence in the New York cohort compared with other cohorts if the virus entered the New York homosexual population before it entered the populations from which the other cohorts were drawn. However, risk of AIDS development in different populations may also depend on the presence of as yet unidentified cofactors.

HUMAN T-LYMPHOTROPIC VIRUS type III (HTLV-III) is the primary etiologic agent of the acquired immune deficiency syndrome (AIDS) (*1*). Surveys of the number of people with antibodies to the virus have suggested that hundreds of thousands of persons in the United States have been infected with HTLV-III, and as of January 1986 more than 16,400 patients with signs and symptoms meeting the surveillance-definition of AIDS have been reported. Because the interval between HTLV-III infection and the development of AIDS may be lengthy, the long-term prognosis following HTLV-III infection is unknown. In the current analysis, we have evaluated the incidence of AIDS following HTLV-III infection in five cohorts—homosexual men in Manhattan, New York; homosexual men in Washington, DC; homosexual men in Copenhagen and Aarhus, Denmark; hemophilia A patients in Hershey, Pennsylvania; and parenteral drug users in Queens, New York.

All five cohorts have been reported in detail elsewhere (*2–8*). Briefly, the homosexual men in Manhattan and Washington, DC, were invited to enroll as consecutive male patients entering the offices of three primary care physicians during May and June 1982, with follow-up approximately on an annual basis through June 1985 (*2, 3*). Follow-up was 90 percent complete at 2 years and 76 percent complete at 3 years. With 6 months of additional tracing, follow-up was 92 percent complete and revealed that one more member of the Manhattan cohort had developed AIDS. Because the subjects followed for the additional 6 months were not actually seen for the study, and because their inclusion in the final results made a negligible difference (<1 standard error), the additional follow-up data are not included in our analysis.

The homosexual men in Denmark were invited to enroll in the study by means of the mailing list of a national (Danish) organization and open clinic sessions in Copenhagen and Aarhus during December 1981 with follow-up sessions in April 1982, February 1983, and September 1984 (*4, 5*). Follow-up was 82 percent complete at the last date. The hemophilia A patients were all those receiving comprehensive care as of September 1982 at the Milton S. Hershey Medical Center in Hershey, Pennsylvania, with follow-up at the semiannual or annual clinic visits (*6, 7*). Their follow-up is 100 percent complete and virtually current. The parenteral drug users were enrolled in late 1981 as part of a case-control study in Queens comparing immunologic parameters in patients hospitalized with soft-tissue infections with those in outpatient methadone detoxifica-

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